1990

Detection of human papillomavirus by nucleic acid hybridization as an adjunct to the papanicolaou smear

Julia Ann Schillinger
Yale University

DETECTION OF HUMAN PAPILLOMAVIRUS BY NUCLEIC ACID HYBRIDIZATION AS AN ADJUNCT TO THE PAPANICOLAOU SMEAR

Julia Ann Schillinger

Yale University

1990

Permission for photocoping or microfilming of "?

"

(Title of thesis)

for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

Signature of Author

1/17/90

Date

Detection of Human Papillomavirus by Nucleic Acid Hybridization

As An Adjunct to the Papanicolaou Smear

A Thesis Submitted to the Yale University

School of Medicine in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Medicine

by

Julia Ann Schillinger

1990

ABSTRACT

DETECTION OF HUMAN PAPILLOMAVIRUS BY NUCLEIC ACID HYBRIDIZATION AS AN ADJUNCT TO THE PAPANICOLAOU SMEAR

JULIA ANN SCHILLINGER

1990

We compared methods for detecting Human Papillomavirus [HPV] deoxyribonucleic acid [DNA] in cervical cytology specimens. Endocervical swabs were obtained from 40 women referred to a Gynecologic oncology clinic for a previously abnormal Papanicolaou [Pap] smear. We employed a modified filter in situ method [ViraPap] for the detection of HPV DNA, followed by HPV typing [ViraType]. Sixteen of 40 specimens were positive for the presence of HPV DNA by hybridization with probes specific to HPV of types 6, 11, 16, 18, 31, 33, and 35. Of these, 5 patients had negative Pap smears but evidence of disease by other parameters (endocervical curettage [ECC], cervical biopsy [CBx], colposcopy). Four of these 5 patients with negative Pap smears were infected with a type of HPV associated with a high risk of developing cervical disease; types 16/18. 7 patients had positive Pap smears with evidence of disease on cervical biopsy, but had negative ViraPap results. We then used the

polymerase chain reaction [PCR] to amplify segments of the HPV-16 E6 and E7 open reading frames [ORFs]. The E6 ORF was found to be the most sensitive. In three independent PCR assays, 13 specimens contained the HPV-16 E6 DNA. These included all of those typed as 16/18 by the ViraType method as well as 5 additional samples not detected by the hybridization methods; 3 with abnormal Pap smears, and 2 with normal Pap smears but evidence of disease by CBx. We conclude that in a population at high risk for cervical disease, the ViraPap can be a useful adjunct to the Pap smear in the detection of disease and that PCR of the HPV-16 E6 ORF may be used as a sensitive, but more labor intensive, confirmatory test.

ACKNOWLEDGMENTS

I would like to extend my greatest thanks and appreciation to Paul Rys and Dave Persing, without whom this project would not have been possible.

When I entered the laboratory to begin this research, I had virtually no lab experience, and little idea of how to design a research project. Paul was an incredibly patient teacher-- his attention to detail, and meticulous technique were a great example to me. He was generous with his time and knowledge, often staying extra hours to see an assay to completion. I am very grateful for all of his help and instruction.

I had no idea when I started this project that I would look back on the experience and be glad of simply having had a chance to work with Dave. In fact, this is how I feel. The creativity with which Dave approaches research, and his boundless enthusiasm for his work were an inspiration to me. He was always available for guidance, yet never intrusive with his advice. He was unfailingly patient and good-natured, and took great care to see that I was given credit for the work I had done.

In addition to all of Paul and Dave's wonderful personal and intellectual qualities it was just downright fun to work with them. The lab was a great place to be-- full of laughter and wit at the same time that serious work was being done. It was a real haven for me during a year that was otherwise somewhat fragmented, and I feel very lucky to have been a part of it.

I would also like to extend my thanks to my thesis advisor Stephen Edberg, for his support of this project, and to Stuart Flynn in the Pathology Department for his interest, and for his contributions of time and energy in rescreening all the Pap smears in this study.

Joseph and Setsuko Chambers very kindly obtained the specimens used in this study, and were available for discussion of the ramifications of using the proposed test.

DEDICATION

I dedicate this thesis to my friends at the Yale School of Medicine who have helped to make these four years a joyous time for me. Medical school is undoubtedly an innately interesting and challenging experience--it is the people with whom one works which make it something more. My close friends during medical school were a great source of strength, humor, and joy during these past four years. I have a great deal of respect and appreciation for them, and I dedicate this thesis to their collective friendship.

TABLE OF CONTENTS

INTRODUCTION

In 1989, 6,000 women died of cervical cancer, and 13,000 new cases of invasive cervical cancer were diagnosed (43). Many studies have established a strong association between infection with the Human Papillomavirus (HPV) and the development of cervical cancer and its' precursor lesions (10, 14, 15, 16, 18, 33, 36, 38, 57, 65, 83); over 90% of cervical cancers examined have been found to contain HPV DNA sequences integrated into the host cell genome (38, 64). There has been tremendous interest and attention focussed on the Human Papillomavirus in recent years as a model for viral oncogenesis. To date more than 60 different types of HPV have been identified. The various types show an apparent cellular tropism, with twenty-three preferentially infecting the genital tract. Among these types there appears to be a range of oncogenic potential.

Current understanding of the biological behavior of cervical cancer suggests that these malignancies are the most severe in a range of precursor lesions usually described as cervical dysplasias. Because the prognosis for a woman with cervical cancer is determined by the stage at which the disease is detected, early diagnosis is the best means of

reducing mortality. The Papanicolaou (Pap) smear has been extremely effective in identifying occult carcinomas and premalignant lesions of the cervix, and the mortality and morbidity associated with cervical cancer have decreased dramatically in the past 40 years. Nonetheless, a percentage of invasive cancers and its precursor lesions go undetected by Pap smears each year. HPV is notorious for confounding the interpretation of Pap smears, blurring the distinction between non-neoplastic proliferative processes and preneoplastic changes, and contributing to ambiguity in the grading of lesions. Increasing recognition of the prevalence of HPV infection in the general population has spurred an interest in a more reliable means of detection.

In 1989 the Food and Drug Administration licensed a test kit for the detection of HPV in endocervical swabs and cervical biopsy specimens. This test, known as ViraPap and manufactured by Bethesda Research Laboratories, utilizes filter hybridization techniques to detect virus in processed specimens. The ViraPap test uses ^{32}P labelled probes composed of RNA sequences complementary to the DNA of HPV types 6, 11, 16, 18, 31, 33, and 35; the seven types most frequently found to infect the genital tract.

This study was designed to assess the clinical usefulness of detecting HPV in specimens of exfoliated cervical cells.

Because screening and diagnostic tests already exist for recognition of the morphologic changes leading to cervical cancer, the usefulness would have to hinge on the tests' ability to provide information the alternative methods could not. In this study the detection of HPV in an endocervical swab specimen by hybridization technique was compared to the results of concurrently taken Pap smears, biopsies, and colposcopy in 40 patients. Because of the apparent range in the oncogenic potential of different HPV types, typing was undertaken on all the specimens shown to contain HPV DNA. Specimens shown to contain HPV DNA of type 16 were confirmed by use of the polymerase chain reaction, a highly sensitive means of DNA amplification.

An assessment of a diagnostic test requires an in depth understanding of the phenomenon being examined, as well as an appreciation for how the proposed test adds to or complements the existing methodology. With these ends in mind, this paper will first address the current understanding of the Human Papillomavirus and its proposed relation to cervical cancer, and then will review the uses and limitations of existing screening and diagnostic tests, with particular attention to the Pap smear and hybridization technique.

The Human Papillomavirus

The Human Papillomavirus is one of the genus Papillomavirus, belonging to the family of viruses known as Papovaviridae, which include the polyomaviruses and vacuolating viruses. Papillomaviruses derive their name from the Latin word 'papilla', meaning nipple or pustule, and the Greek suffix -oma, denoting tumor. This is a befitting etymology for a virus known to cause warts. The viral etiology of warts was first demonstrated in 1905, when Ciuffo demonstrated viral transmission using a cell-free filtrate (10). The first Papillomavirus was described by Richard E. Shope, when he identified the Cottontail Rabbit Papillomavirus as the etiologic agent in infectious Papillomatosis in rabbits (44).

Transmissible Papillomaviruses have now been described in over twenty species of animal, including cows, horses, deer, rabbits, dogs, birds, elephants, and primates, including man. Transmission frequently occurs between members of the same species, a principle which has had major economic ramifications for cattle raisers and for kennel owners who must vaccinate their dogs with a prophylactic vaccine prepared from a suspension of Canine Oral Papillomavirus.

Papillomaviruses from cattle, deer, sheep and Cottontail rabbits can induce tumors when experimentally transmitted to other species, but naturally occurring infectivity between species seems only a slight possibility (8). A unique HPV, type 7, has been identified in the hand warts of butchers and veterinarians. Hybridization studies using probes made from BPV1 and HPV1, 2, 3, 4, and 5, were unable to detect the viral DNA, and restriction analysis showed it to be distinct from known HPVs and BPV1, 2, 3, and 4. Nonetheless, it is possible that this viral type represents an example of cross-species infectivity with an unrecognized bovine, porcine, or ovine type (45).

Classification and Taxonomy

The Human Papillomavirus, like all others of its type, is an icosahedral nonenveloped virus containing only DNA and proteins. Each virion contains a single DNA molecule which is double stranded and circular. The HPV genomes are all approximately 8000 base pairs in length, ranging from around 7200 to 8000 base pairs.

Study of the structure and behavior of the Human Papillomavirus was hampered for many years by the inability to culture infected cells in vitro. The virus infects epithelial surfaces, causing proliferations which are usually

benign, but which can lead to malignant growth. Replication of the virus apparently relies on factors present in differentiating keratinocytes; efforts to create a culture system capable of supporting such growth have not been successful as yet (8). The development of recombinant DNA technology, with the creation of cloned plasmid containing HPV DNA, has enabled researchers to delve into the intricacies of this elusive virus. HPVs have been classified into types and subtypes based on liquid phase hybridization followed by S1 nuclease digestion. If the viruses being compared show homology of less than 50% by this method, the unknown is considered a new type. A subtype is defined as an unknown which shows more than 50% but less than 100% homology with the prototype under these conditions. A reliance on known prototypes clearly limits the ability to identify new, distantly related viral types. It is important to understand that when the nucleotide sequences of different viral types are compared by heteroduplex analysis, far more than 50% homology may be found (10). The complete genomes of HPV types 1, 5, 6, 8, 11, 16, 18, 31, 33, and 52 have been, or are in the process of being sequenced (9). As more extensive sequence data for all the types emerges, a sequence based classification system may prove to be clinically useful.

It has long been recognized that different HPV types have a predilection for infection of different parts of the body.

HPV-1 and HPV-2 typically cause plantar warts on the soles of the feet and hands respectively. Types 6 and 11 are most frequently found in benign exophytic genital warts, or condylomata, on the external genitalia and surrounding the anus. Type 16 and 18 have a preference for the epithelium of the uterine cervix, and HPV type 13 has only been described in the oral papillomas of Heck's disease. These observations have led to a clinical classification of the HPVs by a type-specific preference for different cell types and anatomic locations. The broadest categorization groups the types into those that infect cutaneous surfaces and those that infect mucous membranes. The preference of different types for different locations on the body has been suggested to be an evolutionary response to different types of keratinization within the body (46).

Pathogenesis

Twenty-three of the 60 HPVs identified thus far have been found to infect the genital tract:6, 11, 16, 18, 33, 35, 30, 34, 39, 40, 42-45, 51-59 (9). Of these, types 6,11,16, and 18 are most frequently identified. The sexually transmitted nature of HPV infection was first described in 1954 by Barrett et al.(30). The authors reported on a cohort of 24 women, all of whom had first noticed the appearance of genital condylomata within 4-6 weeks of their respective husbands'

return from a military tour in the Far East. When questioned, all the men admitted to a sexual encounter in the Far East, and all had recently had penile warts. In 1990, HPV infection is acknowledged to be the most common sexually transmitted disease in the United States (84, 87).

The virus is thought to enter the epithelium through any minor abrasions or breaks in the skin. Infection does not usually result when innoculums are introduced onto intact skin (31). The virus infects the basal cells, which rest against the basement membrane in the lowest strata of the epithelium. Apparently the virus requires a cell which is still capable of dividing to initiate infection (8). The basal cells are nonpermissive for vegetative viral growth, and HPV DNA has been detected only in small quantities in the basal and parabasal cells (32, 33). Papillomavirus DNA is detected with increasing frequency in the differentiated cells above the basal layer (33). The virus probably replicates and is assembled in these keratinizing cells, with the infectious virions being shed in the desquamating squames. This model would help to explain why the cervical squamocolumnar junction, with its exposed junction of rapidly proliferating cells, would serve as a likely site of viral entry, and is the site at which around 90% of cervical carcinomas originate (8). This model also supports the notion that the presence of other sexually transmitted diseases which inflame or otherwise

compromise the integrity of the epithelium (herpes, syphilitic chancre, trichomonas etc.), may facilitate infection with viruses such as HPV.

Latent infection may persist by episomal replication in the basal cells, without progression through the keratinizing process (10). HPV DNA has been detected in normal appearing epithelium both adjacent and distant to observed lesions (18, 29, 59). It is not known how long the virus may remain in such a state or what might instigate a productive infection. Spontaneous regression of disease is frequently observed, although whether this a true regression or simply a reversion to latency is not clear.

There are a number of morphologic features associated with HPV infection on histologic examination. The findings associated with productive viral replication are an appearance of hyperplastic proliferations of the basal and parabasal cells (acanthosis), a degenerative cytoplasmic vacuolization (koilocytosis), and variable thickening of the more superficial cell layers (hyperkeratosis and parakeratosis). Other findings include nuclear wrinkling, pyknosis, binucleation, and more severe forms of nuclear atypia including enlargement, irregular chromatin clumping, and hyperchromasia.

Prevalence

The prevalence of genital tract infection with Human Papillomavirus is not known. Estimates vary according to the study design, the subjects and population, the type of specimen selected for analysis (ie. tissue or cytology specimen), and most significantly, the detection method used. Estimates of prevalence rates in the population with normal cytology range from 9% using filter in situ hybridization to 80% using the recently developed polymerase chain reaction (18, 29). Any consideration of prevalence must take the above mentioned variables into account.

Pregnancy

A study examining the prevalence of HPV amongst pregnant women arrived at an rate of 28% (19). In this study cervical smears from 92 pregnant and 96 nonpregnant women were tested for the presence of HPV DNA by Southern blot hybridization and probes for HPV types 6,11,16 and 18. Pap smears taken simultaneously with the sample smear showed no evidence of CIN or HPV infection, and none of the women had a history of a previously abnormal Pap smear. The subjects were age matched and were all sexually active.

HPV DNA was detected in 26(28%) of the pregnant subjects,

and in 12(12.5%) of the nonpregnant subjects, making the detection of HPV DNA 2.3 times more frequent in the pregnant population tested. HPV of type 16 was the type most frequently identified, accounting for 42.3% of the HPV positive smears amongst the pregnant women, but only 25% of the positive smears among the nonpregnant group. When estimates of the amount of viral DNA in a specimen were made, 85% of those from pregnant women contained more than 1.0 pg. of HPV DNA, while the majority of specimens from nonpregnant women (80%), contained less than 1.0 pg. In both groups of subjects the amount of viral DNA in HPV 16 positive specimens was higher than that found in specimens containing other types. The authors draw on these results to suggest that the state of pregnancy allows for the reactivation of HPV and increased viral replication. It must also be considered that an enhanced ability to sample the squamo-columnar junction during pregnancy due to columnar eversion might also contribute to the increased rate of and viral recovery detection.

It is commonly observed that condyloma acuminata often flourish during pregnancy, only to regress in the post partum period (47). Both hormonal influences and an altered state of immunity have been proposed as contributing factors (22). Studies of immunosuppressed or immunodeficient populations also show a higher rate of HPV infection and associated neoplasia. Renal transplant patients on chronic

immunosuppressive therapy, and HIV positive patients form the patient populations for most of these investigations (48, 49).

Epidermodysplasia Verruciformis (EV) is a rare disease with an apparently autosomal recessive pattern of inheritance. It is characterized by an impaired cell mediated immunity, and by widespread skin lesions induced by HPV of specific types. Patients with EV have a high incidence of cutaneous cancers, usually in sun-exposed areas. This disease has served as a model in which to study the interaction between immunologic function and HPV infection (8).

Genomic Organization

Most of the initial work on the genomic organization of the HPV was worked out using the BPV. The details that follow concern the BPV1 genome, but will be related to the HPV. All the Papillomaviruses have the same basic organization; early transcriptional units E1-E8, two late transcriptional units L1 and L2, as well as a long noncoding region also known as the upstream regulatory region (URR). The open reading frames (ORFs) of the Papillomavirus are all located on the same DNA strand and show considerable overlap. Some function has been localized to each of the 8 ORFs, with the exception of E3. The E3, E5, and E8 ORFs are not as highly conserved as other portions of the genome and may not be present in all

Papillomaviruses. Over 90% of the genome is accounted for by coding regions.

The transforming (early) segment of the BPV1 genome contains the eight ORFs, E1-E8. The E1 ORF is the largest of the eight and overlaps slightly with E2. It has the capacity to encode a polypeptide of 68 kilodaltons (K). This protein has been shown by mutational analysis to have a critical role in episomal replication; cells transformed by virus selectively mutated in the E1 ORF show Papillomavirus integration (50). The E2 ORF encodes a 45K polypeptide, and overlaps completely with E3, which lacks its own start codon, and must be spliced to an upstream exon if it is to be expressed as a functional polypeptide. The BPV E4 also overlaps with E2 and codes for a 12K polypeptide. Both the E2 and E4 proteins appear to be involved in the transforming process. The E5 is found at the 3' end of the early region. It encodes a very hydrophobic polypeptide, a 44 amino acid membrane-associated transforming protein. The E8 ORF has a variable location in different Papillomaviruses and is probably not functional. The E3 and E5 ORF's are not found in all the Papillomaviruses.

The E6 and E7 ORFs are found at the 5' part of the transforming region where they overlap slightly. The two code for polypeptides of 16K and 14K respectively. These gene

products seem to be important in transformation; Harlow et al. showed that the HPV16 E7 gene product, a nuclear phosphoprotein, binds to the retinoblastoma protein, the product of a cellular antioncogene (13). In addition, the HPV16 E7 protein can cooperate with ras to transform baby rat kidney cells and transactivates the adenovirus E2 promoter (51).

The L1 and L2 ORFs of the late region code for polypeptides with predicted weights of 55.5K and 50.1K respectively. Both these ORF's code for proteins present in the Papillomavirus capsid, although L1 appears to code for the majority of the protein, VP1 (8). The noncoding or upstream regulatory region has several portions which are AT rich, and several nearly perfect palindromes. It is presumed to contain control elements for DNA replication and transcription (8).

As mentioned earlier, the Human Papillomaviruses have an organization very similar to the Bovine Papillomaviruses. When compared to HPV1, the E8 ORF was not in a corresponding position, although the other ORFs were in equivalent locations. Portions of the E1, E2, L1 and L2, contained notable homologies. Within the E6 and E7 ORFs, although there was less homology, there were certain common characteristics. These common findings were also true of HPV6. For instance, the amino acid sequence Cys-X-X-Cys is repeated four times in

the predicted E6 polypeptide of both genomes (BPV1 and HPV1a). In the E7 ORF the same motif is repeated twice in both genomes. There are also similarities in the noncoding regions.

Transforming Properties

The HPV detected in malignant lesions is usually in an integrated form, while that detected in benign lesions is typically in an episomal state (10, 41). HPV 16 and 18, which together account for more than 90% of all the HPVs identified in cervical tumor specimens, are usually found to be integrated in higher grade lesions and carcinomas, while other lower grade lesions harbor HPV DNA in episomal form, with proportionately more infections with types 6 and 11 (8, 38, 41, 88). Several immortal cell lines, Hela, SW756, C4-1, SiHa, and Caski contain integrated HPV DNA and are derived from cervical carcinomas. The first three contain HPV type 18, and the latter two, DNA of type 16 (8). It appears that integration results in a deregulation of cellular controls, however, integration does not necessarily lead to transformation; several benign lesions have been shown to contain integrated HPV DNA, and rarely a cervical carcinoma will contain HPV DNA in episomal form (52). It is possible that integration is not the transforming event, and rather, that transformed cells are somehow permissive of integration.

During integration the E1, E2, and E5 as well as the late ORFs are disrupted. This may lead to deregulation of the E6 and E7 ORFs, which remain intact during the integration event. The upstream regulatory region is also reliably conserved despite integration. Both the E6 and E7 ORFs undergo transcription and translation into viral proteins and appear to be required for the transforming properties (35, 53). As mentioned above, the E7 protein has recently been shown to bind a protein product of the Retinoblastoma gene, a model antioncogene. When inactivated, the RB protein is effectively eliminated from the cell, and increased cell proliferation and oncogenesis may be allowed (34).

A recent paper discussed the differences between the E7 protein of HPV type 16, associated with a high percentage of cervical carcinomas, and type 6b, a nononcogenic type (15). The E7 proteins differed in a number of ways. First, they had different electrophoretic mobilities in SDS-PAGE; type 6b resolved into three distinct species, the slowest of which was phosphorylated. Second, the HPV16 and 6b E7 proteins had different sedimentation properties in nondenaturing glycerol gradients. Third, and most significant, the E7 proteins of these two types had different capacities to bind the RB protein; the HPV6b product bound much less extensively than the type 16 product. Apparently binding only occurred with the

phosphorylated species of type 6b. These findings suggest that the oncogenic capacity of various types of HPV may be partially determined by the affinity of the E7 gene product for the RB protein. The authors postulate that cellular changes such as enhanced activity or higher levels of kinase in host cells containing HPV6b could lead to more effective binding of the RB protein, and more effective oncogenesis. This may account for the rare finding of type 6b in malignancies (34).

Human Papillomavirus and the Association with Cervical Cancer

The epidemiology of cervical cancer suggests an infectious,
sexually transmitted agent. There are several pieces of evidence to support this model. First, cervical cancer is rare in nuns and sexually abstinent women (54, 55). Second, a positive association exists between the development of cervical cancer and early age at first coitus, multiple sexual partners, and the presence of other sexually transmitted diseases (56). Third, a woman whose husband has been previously married to a woman with cervical neoplasia, has herself a four-fold greater risk of developing cervical cancer (57). Fourth, an increased incidence of cervical cancer has been documented in the wives of men with penile carcinoma (58). Fifth, the incidence rates of cervical and penile

carcinoma are proportional, even across wide variations in incidence.

Until recently the Herpes Simplex Virus 2 (HSV-2) was considered a likely etiologic agent for cervical cancer. This notion was based primarily on seroepidemiologic studies which consistently showed patients with cervical cancer to have higher HSV-2 antibody titers than matched controls, and also on the demonstrated oncogenic potential of partially inactivated HSV-2 in rodent cells. Using DNA-RNA hybridization techniques some workers have found HSV RNA in malignant and premalignant cervical tissues, but in only one case has HSV-2 DNA been detected in a cervical cancer specimen (86).

The demonstration of HPV DNA in the majority (90%) of cervical cancer specimens, as well as in 75- 95% of CIN lesions of all grades (CIN1-3), suggests that HPV may be a cofactor in the development of this disease (35, 38, 64). HPV DNA has also been detected in other genital tract tumors (17, 39, 60). In a recent series of 53 Penile carcinomas, 27/53 (51%) were found to contain integrated HPV DNA. 26 of the 27 were type 16 and one specimen was type 18 (39). The authors suggested that this might be an underestimation of the actual presence of HPV; when two tissue samples from a known 16/18-positive specimen were analyzed, only 33% were positive in

both samples. Another study examined 16 vulvar carcinomas for the presence of HPV DNA (17). Fourteen of 16 (88%) contained HPV DNA, 13/16 (81%) type 18. HPV has also been detected in carcinomas of the vagina, and anus (60). The recent attention focussed on HPV as an oncogenic agent has led investigators to look for the virus in malignancies outside the genital tract (40).

HPV also exists in a multitude of benign lesions, most notably condyloma accuminatum. The DNA can also be found in normal epithelium in genital areas of patients with condylomata, and in the normal cervix (61, 59). There seems to be a range in oncogenic potential amongst the HPV types, with type 6 and 11 representing a more benign agent, and types 16 and 18, seemingly more aggressive forms of the virus. A recent review of four studies demonstrated an inverse relations ship between the detection of types 6 and 11 and increasing grade of cervical intraepithelial neoplasia, and a positive association between types 16 and 18 and increasing grade (10, 64). Type 16 is the HPV most commonly found in cervical neoplasias and CIN lesions. It has been suggested that type 18 is found more frequently in adenocarcinoma of the cervix (62). Southern blot analysis of 11 primary adenocarcinomas detected HPV18 in 5 (45%) of the tumor specimens, and type 16 in 2 of 11 (18%) (36). It has been proposed that type 18 is associated with approximately 35% of

cases of a "rapidly progressive cervical cancer," defined as cervical cancer occurring within three years of the last true negative Pap smear. This type of tumor affects a population which is distinguished by younger age, higher socioeconomic class, more advanced disease at time of diagnosis, and greater reported frequency of benign gynecologic conditions (uterine leiomyomata, vaginitis) when compared to a control cervical cancer group (63). However, when Koutsky et al. conducted a review of ten studies of squamous cell carcinomas and seven studies of adenocarcinoma, HPV type 18 was detected in 32/281 (11%) of the squamous carcinomas but only 6/31 (19%) of the adenocarcinomas (10). This phenomenon clearly requires further investigation, and control for sampling error (see section on Pap smear collection).

On the basis of broad epidemiologic studies of the association between different types of HPV and cervical lesions of all grades, types 16 and 18 can be considered "high risk" for carcinogenesis and types 6 and 11 can be considered "low risk." Types 31, 33, and 35 may be viewed as "intermediate-high risk" (64). Assignments of associated risk are valuable as general prognostic indicators, but it is important to realize that types 6 and 11 have been identified in some cervical cancers and types 16 and 18 do exist in normal epithelium without evidence of disease (64, 88). Thus, infection with any of the types of HPV should be considered

to carry some risk of carcinogenesis. Coinfection with more than one viral type has been reported to occur in between 3 and 20% of cases of infection (66). It has been proposed that coinfection may result in a synergistically enhanced progression of dysplasia. It is also possible that recombination occurs between different viral types, resulting in a more oncogenic form.

A patient at high risk for cervical neoplasia has been described in the literature (56-58). Early age at first coitus, early age at first pregnancy, multiple sexual partners, a partner with penile condylomata or penile cancer, and a history of an abnormal Pap smear can all be considered risk factors. A history of HPV infection, infection with other sexually transmitted diseases, and immunosuppression or deficiency have all been linked to the development of cervical disease.

In addition, several cofactors have been suggested for the oncogenesis of HPV on the cervix. Among these are HIV, HSV, GC, Chlamydia, bacterial vaginoses, bacterial metabolites, and mutagenic degradation products. Cigarette smoking, vitamin deficiencies, and the use of oral contraceptives have also been proposed (10).

The association between cervical cancer and early age at

first intercourse, early age at first pregnancy, and the use of oral contraceptive agents may all be explained by the fact that the transformation zone (squamocolumnar junction) is most exposed during adolescence and in excess estrogenic states (ie. pregnancy and oral contraceptive use), therefore creating an easy venue for viral entry. It is also possible that the increased prevalence in populations with these "risk factors" is a result not of increased rates of infection, but rather, of increased rates of detection due to columnar eversion and enhanced sampling, and is a truer estimate of prevalence.

As discussed earlier, HPV DNA exists in episomal form in condyloma accuminatum and in all grades of CIN. When cervical carcinoma specimens are examined, the DNA is found to be present in an integrated form. The eight immortal cell lines described earlier also all contain HPV DNA in its integrated form. Integration is currently believed to be a critical and determinant step in malignant transformation by DNA animal viruses in vitro and in tumor production in vivo. The E6 and E7 ORFs are always maintained intact during integration, and code for proteins whose transforming properties are presently being investigated. The fact that HPV DNA exists in the integrated form in cancer specimens is strong evidence in support of a causal relationship.

There are, of course, several possible explanations for

the close association between HPV and cervical cancer other than a direct causal relationship. It is possible that HPV is simply an eager opportunist of cell dysgenesis, or is made more easily detectible by dysplastic change. Perhaps integration is permitted only by a transformed cell, and the integrated HPV DNA represents a result rather than a cause of cell transformation. Despite these explanations, at present there is substantial evidence in support of a etiologic role for HPV in cervical carcinogenesis.

The Pap Smear

In 1928, George Papanicolaou and Aureli Babes, a Rumanian pathologist, independently introduced the notion of using exfoliated uterine cervix cells found in the vaginal pool as a means of detecting cervical cancer. Over the next two decades Papanicolaou furthered his studies of this cytologic phenomenon and in 1943 he published the monograph, Diagnosis of Uterine Cancer by Vaginal Smear. In 1947, J. Ernest Ayre, a Canadian gynecologist, determined that a sample of cervical cells obtained by direct sampling from the cervix with a wooden spatula produced a smear which was more efficient and simpler to examine (67). Hence the Pap smear and one of its' implements, the Ayre spatula, were born.

The use of the Pap smear to sample cells from the uterine

cervix, with subsequent interpretation by a cytologist, has enabled physicians to detect occult cervical carcinomas and its precursor lesions. Because the prognosis for a patient with cervical cancer is determined by the clinical stage of disease at the time of diagnosis, the Pap smear has assumed a highly important role as a cancer screening test. In the United States, mortality from cervical cancer has decreased over 70% in the past 40 years (43). This dramatic improvement can be attributed in large measure to the use of the Pap smear, leading to detection of cancers at earlier stages of disease, and to the regular visits to the physician that accompany the use of this screening test.

Despite striking improvements in early cancer detection, there are many flaws in the application of the Pap smear. The use of the pap smear has never been evaluated in a prospective fashion for obvious ethical considerations. Nor has the efficacy of different screening intervals been subjected to prospective analysis; different medical organizations have advised different schedules for routine screening. The use of a varied nomenclature for describing cervical cytopathology has contributed to confusion in the referring physician's interpretation and management of potentially precancerous lesions. Finally, false negative readings of abnormal pap smears and poor reproduceability of interpretation, even by a single reviewer, has plagued the test.

It is not unusual for a patient diagnosed with cervical cancer to have a recent history of normal Pap smears (3, 71, 85). In one study of 264 women who were evaluated and treated for primary epithelial carcinoma of the cervix (64% had stage I disease), 97 women (37%) had a history of a normal pap smear within three years of diagnosis; of these, 48 (18% of the total) had had the normal smear within the past year. Twenty-one of the patients presenting with primary epithelial carcinoma (8%) had never been screened, and the results of previous pap smears were not available for 81 (31%) of the women. Finally, 8 of the women had normal Pap smears at the time of histologic diagnosis of cancer (3). This study was designed to assess the impact of different Pap smear screening intervals on the prevention of advanced disease. Nonetheless, it highlights the potential weaknesses in a reliance on the Pap smear as a screening test. There are a number of reasons that the Pap smear is not an infallible screening test. Some of these relate to the very nature of cervical cancer itself, and others to the collection, interpretation, and reporting of results.

Specimen Collection

The reliability of cervical cytology specimens is largely dependent on the skill of the clinician collecting the

specimen, the area sampled, and the method of specimen collection used. The majority of cervical squamous carcinomas arise at the squamocolumnar junction, or transformation zone. A mechanism for preferential infection of this portion of the cervix by the human papillomavirus has been proposed (see earlier text). In adolescents this zone is well exposed on the vaginal portion of the cervix, but as a women ages this cell junction moves rostrally, into the cervical os. Consequently, an adequate sampling of this region becomes more difficult in older women. A number of studies have correlated the absence of endocervical cells with a lower rate of detection of malignancy (68). It is generally accepted, therefore, that a Pap smear that does not include endocervical columnar cells does not represent a sampling of the transformation zone, and is unsatisfactory.Using this criteria, as high as 30% of all gynecologic cytology specimens received by some laboratories would be considered inadequate (69). It is possible that the so-called "rapidly progressive carcinoma", is a result of a type specific predilection for endocervical cells. While the cellular tropism of this particular HPV type remains unestablished, if in fact the virus infects the columnar cells of the endocervix, it may be escaping early detection because of poor sampling as described above.

A recent development which may greatly enhance specimen

collection is the use of the endocervical "cytobrush" in conjunction with the Ayre spatula. In one study of 5716 Pap smears which were obtained using an Ayre spatula and cytobrush, 98% were found to contain endocervical cells. This was compared to 24,496 slides which were obtained with spatula alone, only 84% of which included endocervical cells (69). Another study compared the use of cytobrush and spatula to specimen collection by spatula and cotton swab. When 510 slides prepared in each manner were examined, 12% of those using the spatula and swab lacked endocervical cells, while only 1.7% of those utilizing the cytobrush lacked these cells (5). Potential drawbacks to the use of the cytobrush include the need to reorient cytopathologists to interpreting the slides which can contain whole sheets of endocervical cells, as well as revealing an increased number of minor cytologic atypias. The abrasive effect of the cytobrush in the endocervical canal can also produce more bleeding than the commonly used cotton swab, and the resultant blood can obscure cells on the Pap smear. Another means of sampling cervical and vaginal cells is cervical aspiration, and cervicovaginal lavage (26).

Because the Pap smear consists of only a sampling of cells, it cannot be considered truly demonstrative of the histologic state of the cervix. Even when Pap smears have been properly collected, there may be precancerous lesions on the

cervix which are represented only by a few atypical cells. Different lesions shed cells in a different fashion, and may be over or under represented on the Pap smear. In invasive cancer, the outer surface of the lesion can become necrotic, yielding a Pap smear obscured by debris, and failing to show any obvious cellular abnormalities. It has been suggested that all patients in a high risk group (such as adolescents at a sexually transmitted diseases clinic) be given routine colposcopy at the time of Pap smear (anecdotal). Lesions which might escape detection by Pap smear would be recognized, and could then be confirmed by biopsy specimen. However, colposcopy is a time consuming procedure, which must be performed by highly trained personnel--the implementation of routine colposcopic screening would make for an expensive and time consuming evaluation in clinical settings already overburdened by patient load. Patients undergoing colposcopy, while at a high risk for cervical disease, would also have a greater likelihood of more innocent genital tract infections which could present falsely positive findings on colposcopy, This would lead to biopsy, thus generating a lot of additional pathologic material requiring interpretation. Also of note, the colposcopic examination is far more uncomfortable for the patient than a simple speculum exam and Pap smear. Patient compliance is already an impediment to annual screening by Pap smear alone; if routine colposcopy was added to annual checkups, compliance could decrease further.

Filter hybridization for HPV DNA has also been suggested as a screening test for patients at high risk for cervical neoplasia. This possibility will be discussed extensively in the following section.

Cytopathologic Interpretation

The evaluation of Pap smears is a poorly standardized and regulated process, without extensive and effective quality control. Recent reports in the popular press (Wall Street Journal, November 1, 1987), focussed on the Pap smear "industry", where the number of Pap smears which could be evaluated by a cytotechnologist within a certain time frame was largely unregulated. The presumption is that this results in an increased risk of abnormal Pap smears being read as normal. There has been little study of the time dependency of an accurate Pap smear evaluation, but it is generally believed that a thorough screening of a single slide requires at least five minutes, and that the recognition of cytologic abnormalities would suffer were less time allotted.

Before issuing a license for interstate commerce, the Centers for Disease Control requires that a laboratory observe a quality control measure whereby 10% of all Pap smears read as negative be rescreened. Given the low rate of abnormal

smears (approximately 10% of all smears taken), this method could fail to detect inadequate evaluation. There are other quality control efforts made by dedicated laboratories. A histopathologic correlation can be made when biopsies have been taken, and a review of a patient's previous pap smears is often undertaken when a current smear is evaluated as questionable or abnormal. The 10% rescreening measure remains the only regulated quality control measure. The implementation of other measures is at the discretion of a particular laboratory. Only a few states require proficiency examination by those reading cytology. In New York, a competency examination has been required of personnel reading Pap smears. An analysis of a fourteen year period demonstrated a significant improvement in the performance of the pathologists (70).

There are clearly many improvements which could be made in the evaluation process at an administrative and regulatory level. At the foundation of these problems rests the fact that even when performed by expert pathologists, the interpretation of a Pap smear is extremely difficult. As described above, a sample of cells may not reflect underlying disease, and at best, the interpretation of cellular changes is a subjective process.

A false negative Pap smear is one read as normal despite

the presence of disease. As discussed, the failure to detect disease can be a consequence of tumor behavior, sample collection, or smear interpretation. A review of five studies using a comparison of two smears to assess false negativity, found rates to range between 0.0% and 29.7% (71-75). Sensitivity rates calculated from these same studies ranged between 77.1% and 100% These studies were based on populations from whom both an endo and ectocervical specimen had been collected, and in whom disease was diagnosed within three years of the "negative" smear. Smears defined as false negative were those which were found to represent sampling or interpretive errors when reevaluated following a subsequently abnormal pap smear. These results are confounded by the fact that different instruments were used in specimen collection, the fact that cervical lesions can regress, persist, or progress, and that some cervical lesions will continue to be undetected even by the second Pap smear. Nonetheless, these studies confirm the fallibility of the Pap smear as a cancer screening test.

Several studies have addressed the issue of inter-observer variability in the interpretation of Pap smears. One study investigated 339 cases of tissue proven cervical malignancy (carcinoma in situ, invasive squamous carcinoma, endocervical adenocarcinoma, and lymphoid malignancy involving the cervix) (25). A total of 66 (20%) of patients had had a

Pap smear interpreted as normal within a year of diagnosis. These cytology slides were obtained and rescreened by two pathologists who had no previous knowledge of the present diagnosis; "true negative" slides were interspersed amongst the study slides as a measure of internal control. The reviewers assigned the slides to one of three false-negative categories; (1)Sampling error--no malignant or dysplastic cells seen on review, (2)Screening error--malignant or dysplastic cells present but not recognized by the cytologist, and (3)Interpretation error--dysplastic or malignant cells marked by the screening cytologist but not interpreted as significant by the overseeing cytopathologist. Overall, these Pap smears represented a 20% false negative rate for the detection of malignant disease. After rescreening, sampling error was thought to account for 62% of these cases, screening errors accounted for 16% and interpretation accounted for 22% of the false negatives. For the purposes of this discussion "screening" and "interpretation" errors can be considered together, as they represent a failure on the part of the personnel responsible for recognizing malignancies. When these errors are summed, interpretive errors account for 38% of the false negatives. It should be noted that slides which the reviewing pathologists considered "unsatisfactory" were attributed to interpretive error, rather than being included in the "sampling error" group. Slides considered unsatisfactory contained few cells, had drying artifacts,

obscuring inflammation or necrosis, or showed heavy clumps of cells; slides which did not contain endocervical cells were not considered unsatisfactory.

Another study by Horn et al. concerned the reliability of the characterization of HPV infection on a Pap smear (23). Two pathologists well experienced with the cytologic manifestations of HPV infection were asked to read 87 slides, a proportion of which were known to contain evidence of HPV infection as described by Meisels criteria (24). Approximately 25% of these slides were then reintroduced into the screening system in an attempt to assess intraobserver variability in interpretation. The pathologists classified the smears on the basis of (1) the presence of absence of HPV infection, and if HPV was thought to be present, (2) the degree of certainty with which the diagnosis of HPV was made.

The two pathologists agreed on the presence of absence of HPV infection 74% of the time. The kappa statistic was used to control for agreement which could be expected by chance alone; the value 0.38 reflects only a fair degree of agreement, but far more than would be expected by chance alone. Intraobserver reliability was found to be 96% for one pathologist, and 79% for the other.

It is very significant that in Horn's study. Twenty

percent of the slides were considered to be borderline or questionable diagnoses of HPV, ie. one pathologist classified the slide as lacking evidence of HPV, and the other considered some evidence of infection to be present. In their paper on the cytologic patterns of condylomatous lesions of the cervix and vagina, Meisels et al. lament the ambiguity of interpretation of cytologic findings associated with HPV infection, and express concern that these findings are often misinterpreted as CIN1 (24). In light of the unreliability in detecting HPV, and difficulties in interpreting the findings when present, a more sensitive and reliable means of identifying HPV infection is needed.

Two recent studies examined both inter and intra-observer variation in the interpretation of cervical intraepithelial neoplasia in histopathological specimens. Analysis was conducted using the Kappa statistic; both studies found overall poor interobserver agreement. One study found agreement between reading pathologists was best on immature squamous metaplasia and CIN3, and that there was only poor agreement on CIN1 and CIN2 (7). Importantly, it was found that the ability to distinguish morphologic changes typically associated with HPV infection resulted in the poorest degree of agreement in the study; little more than what would be expected by chance. It was concluded that HPV may induce morphologic changes which may exaggerate the apparent severity

of dysplastic changes in cervical epithelium. Analysis of intraobserver variation also showed poor agreement, but this parameter was assessed with unusually difficult cases, and may reflect some selection bias. The second study found that agreement was best with regard to invasive lesions, mediocre in grade III lesions, and poor for lesions of grade I and II (42). The results of this study also revealed that even experienced histopathologists had difficulty distinguishing reactive proliferations of the epithelium (including changes due to the human papillomavirus), from CIN1.

The use of the term "atypia" is often used to describe Pap smears.In theory, this diagnosis is made when the epithelial cells show slight to moderate alterations in nuclear size and morphology. These changes can include a nucleus 1.5-2 x normal size, binucleation, (finely granular and evenly distributed chromatin, chromacenters) and an indistinct perinuclear halo in an orangeophilic cytoplasm. There has been a great deal of controversy surrounding the appropriate management of patients with such findings. Several studies have shown that there is a high rate of neoplasia in patients with such smears (77-80). In one study, 406 women with "atypical" pap smears were referred for repeat Pap smear and colposcopic evaluation within six 6 weeks to 3 months of the original Pap smear (1). Biopsies and endocervical curettage were performed if appropriate. In 18.7% of the patients, CIN was documented

histopathologically at this return visit. While these findings may partly reflect problems with sample collection and the ability of cervical lesions to progress in severity over time, they also reflect the ambiguity in the interpretation of cervical smears, and the danger inherent in a vague classification system.

Diagnostic Reporting Systems

Three methods of describing cervical cytology specimens are currently in use and a new system has recently been proposed (6). The Papanicolaou classification (class I-class V) system is based on the certainty with which a cytopathologist can describe a smear as containing malignant cells. It does not provide for description of non-cancerous findings. The World Health Organization developed a system for describing Pap smears along a continuum from "normal", to "invasive squamous carcinoma," with the premalignant lesions designated as mild, moderate, or severe dysplasia. Pap smears have also been reported as ranging from normal, through grades of cervical intraepithelial neoplasia (CIN1, 2, and 3) to invasive squamous cell carcinoma. Both of the latter two systems had the advantage of describing the findings in morphologic terms, but years of experience showed that there was a great deal of variability in the assignation of these terms, and poor reproduceability among readers. In an effort

to minimize the ambiguity of the terminology, and to reduce the confusion generated for clinicians using these reports for clinical management decisions, the participants of a National Cancer Institute Workshop held in December, 1988, proposed a new system for reporting Pap smears. This system, known as the Bethesda System, classifies all precancerous lesions as "Low grade Squamous Intraepithelial Lesion" (SIL), and "High Grade Squamous Intraepithelial Lesion." These terms encompass the descriptive terms which have been used previously. Low grade squamous Intraepithelial lesions are those showing cellular changes associated with HPV infection, and mild dysplasia (CIN1). High grade SIL encompasses morphologic changes previously described as moderate, and severe dysplasia, and carcinoma in situ.

The Bethesda system limits the use of the term "atypia" to describe those findings which are of "undetermined significance." Specific mention was made in the report from the NCI workshop that the term not be attached to otherwise defined inflammatory, preneoplastic, or neoplastic cellular changes. The guidelines for cytologic description include all these categories. The workshop also recommended that a cytopathology report provide a statement on the adequacy of the specimen.

Detection Methods

There are a number of means of detecting HPV infection of the genital tract. Some of these rely on morphology and are used clinically, and others use nucleic acid hybridization and other molecular biology techniques to recognize the presence of the virus.

Gross Inspection

Condyloma acuminata can usually be detected by gross inspection of the genitalia. An application of 3% acetic acid and the use of a magnifying hand lens will reveal more subtle lesions. Raised, condylomatous lesions are typically found in the region of the external genitalia, but are often in the mucous membranes of the anus, vulva, introitus, and vagina, and sometimes appear on the cervix. The fact that HPV DNA has been demonstrated in normal appearing epithelium clearly illustrates the limitations of gross examination.

Colposcopy and Cerviography

HPV-associated lesions on the cervix are often invisible to the naked eye, but can be seen with colposcopic examination after the application of acetic acid. Such lesions can be recognized by their white appearance, often with a slightly

raised, mosaic, or punctate pattern.

Cerviography is a recently developed diagnostic method which combines the principles of colposcopy with a simple photograph of the cervix. The cervix is photographed after application of 5% acetic acid: abnormal cervical patterns can be recognized in the photographs by trained reviewers. A comparison of the Pap smear and cerviogram found the cerviogram to be significantly more sensitive, but less specific than the Pap smear for the detection of CIN lesions (76). As with other means of detection, the inability to detect nonproductive (latent) lesions with these techniques limits their usefulness. In addition, both colposcopy and cerviography require highly trained personnel to carry out the examination and interpret the findings.

Light Microscopic Examination of Cytologic and Histologic Specimens

As has been discussed in some detail, pathologists examining cytology and tissue specimens using light microscopes have defined characteristic morphologic changes associated with HPV infection. The koilocyte is considered pathognomonic, but viral changes are often difficult to detect in the higher grade lesions of CIN, and there is substantial interobserver variation in the identification of viral

associated findings (23).

Electron Microscopy

The electron microscope has been used to examine specimens for the presence of HPV viral particles. Although particles of the appropriate size and structure have been identified in the cells of condyloma accuminatum, flat condylomas, and dysplasias, they have only been seen in 10-50% of the specimens thought to show evidence of HPV infection by clinical, cytologic, or histologic criteria. In addition to the apparently low sensitivity of this method, it is a time consuming process requiring a skilled technician (81, 82).

Immunohistochemistry

Immunohistochemical techniques have been applied to testing for HPV. Anti-Papillomavirus antibodies have been raised in rabbits; the antiserum is commercially available and reacts with VP1, the major capsid protein encoded by the L1 ORF of the Papillomavirus. Unfortunately, this technique is only able to detect capsid protein in approximately 50% of histologically positive specimens, and is limited to the detection of productive lesions. Because the L1 gene product is expressed primarily in the koilocyte, the antigen becomes less detectible in the progressively higher grades of CIN as

koilocytes themselves are fewer in number. In addition, because commercially available antiserum is produced against BPV1 virions, different HPV types do not react equally well; for instance, BPV1 and HPV6 share more homology in the genus specific epitope of VP1 than BPV1 and HPV16. "the genus specific epitope of the major capsid antigen is more closely conserved between BPV1 and HPV6 than between BPV1 and HPV16" (10). Thus, HPV16 is less detectible using this antisera. Antibodies to other viral proteins such as early region gene products will greatly enhance the sensitivity of this technique (10).

Hybridization

Nucleic acid hybridization has proven to be a far more sensitive means of detecting HPV DNA than any of the methods discussed thus far. A multitude of studies have documented the detection of HPV DNA in specimens with normal cytology using hybridization techniques (18, 19, 20, 25, 26, 27, 28). The use of these methods has added enormously to the understanding of the epidemiology and pathogenesis of HPV.

Southern Blot Hybridization

Southern blot hybridization is generally accepted as the most sensitive of the hybridization tests, and is considered

the "gold standard" for typing studies. Briefly, total cellular DNA is extracted from a specimen, and cut with a restriction enzyme known to have multiple restriction sites within the HPV genome. The resultant fragments are separated by electrophoresis on an agarose gel, denatured, neutralized, and transferred to a nitrocellulose or nylon filter according to a method described by Southern (83). The filter is then hybridized with radiolabelled probes complementary to the DNA sequence being sought. Autoradiographic exposure will reveal a series of fragments of different lengths.

Southern blotting is considered highly sensitive, capable of detecting as few as 0.1 copies of HPV DNA per cell when using 10 micrograms of cellular DNA as starting material (11). The procedure can be carried out with conditions of low and high stringency, and can thus be used to screen for the presence of HPV DNA and then used to test for more specific typing information. Human Papillomaviruses of different types will produce a characteristic pattern when cut with specific restriction enzymes. This has obvious applications for typing assays, but it also has the more general advantage of confirming that a positive result is in fact due to the virus in question, and not to nonspecific hybridization. The pattern of the restriction digest also permits differentiation between integrated and episomal forms of the virus, and allows for the identification of particular genomic fragments.

One of the disadvantages of this detection method is that it requires a relatively large amount of DNA. Such quantities of DNA can be recovered from a biopsy specimen, but less reliably from a cervical cytology specimen, thus necessitating more invasive sampling. The procedure is time consuming and labor intensive, and does not provide any information concerning the localization of HPV DNA within the specimen. Finally, the assay requires fresh tissue, and cannot be used for retrospective analysis.

In Situ Hybridization

In situ hybridization techniques probe tissue or a smear directly, without disrupting the normal tissue or cellular architecture. DNA or RNA probes can be generated, and can be made type-specific. The great advantage of this technique is that it provides information on the location and frequency of HPV infection within a specimen. This method enabled researchers to identify HPV sequences in normal and dysplastic tissue contiguous with cancerous regions of cervical tissue (84). It has also been possible to demonstrate the paucity of HPV sequences in the early basal and parabasal cells at the basement membrane, and the much higher copy number seen in more fully differentiated keratinocytes (33). Small samples are adequate for testing, and the technique can be applied to

paraffin embedded specimens, enabling retrospective study. In situ hybridization can also be used to detect mRNA, enabling the one to detect the expression of specific viral genes.

Despite the advantages of in situ hybridization, its' principle limitation is that it is not as sensitive as other detection methods. In specific instances where only a few cells in a lesion harbor the HPV DNA, this method of detection may be more sensitive than techniques such as Southern blotting which essentially average the amount of HPV DNA present in a sample. In general, however, this method is not nearly as sensitive as other means, especially in frankly neoplastic specimens (19).

Filter In Situ Hybridization

Use of the filter in situ hybridization bypasses the time consuming DNA extraction process; cells collected by smears or cervical scrape are applied directly to a nylon membrane. After application, the cells are chemically disrupted, the DNA denatured, and the filter is probed with complementary radiolabelled or biotinylated nucleic acids. This is a very fast, simple means of detection which can be easily applied to large screening studies. Only a small amount of DNA is required.

This technique has a number of limitations. First, the results of this test provides no information on the localization of DNA sequences within the specimen. Like the Southern blot, it is essentially a DNA averaging assay. Second, non-specific signals from cellular debris may give false positive results (10). Third, a higher rate of dual infection has been reported using this technique; high levels of HPV of one type may result in cross reaction with another type-specific probe (10). Last, while superior to the in situ method, the sensitivity of this detection method is less than that of the Southern blot(19).

Slot Blotting

The slot, or dot blot method can be considered a hybrid of the Southern blot and filter in situ methods. First total DNA is extracted from the specimens. The DNA is denatured, neutralized and applied to a nitrocellulose or nylon filter using a slot or dot blot manifold. This apparatus effectively concentrates the applied DNA into a defined area. Labelled probes can then be hybridized to the filter. The slot blot method has a sensitivity comparable to the Southern blot when RNA probes are used (85). It is estimated that 1 virus/10 host cells could be detected by this method (20). Only a small amount of DNA is required for this assay;this quantity is

easily provided by a cervical cytology specimen. The technique is readily applicable to large screening studies.

Because of the possibility of false positives and nonspecific hybridization, this test must be carried out under highly stringent conditions. As with the filter in situ technique, specimens with large quantities of HPV DNA of one type may cross react with probes directed against a second type. Like the other techniques making use of DNA extraction, slot blot technique does not yield any information about the distribution or localization of HPV DNA in the specimen.

The ViraPap Detection Kit

The ViraPap Human Papillomavirus Detection Kit represents the first hybridization technique to be developed into an FDA approved detection kit for HPV. The method used in this study is essentially a modification of the filter in situ hybridization technique. Samples are collected from the endocervix using a dacron tipped swab and the exfoliated cells are stored in a transport media. The cells are chemically disrupted and the DNA denatured, and the solution is applied to a nylon membrane using vacuum filtration through a dot blot manifold. ^{32}P labelled HPV RNA probes prepared by transcription from recombinant plasmid are then incubated with the membrane and hybridization occurs between the

membrane-bound sample DNA and the complementary RNA probes. The kit provides two positive controls which are dilutions of cells from the Hela cell line known to contain integrated HPV18 DNA, and a negative control of HTB-31 cells which contain no HPV DNA.

The ViraPap test has been evaluated in comparison to Southern blotting technique by the manufactures. In a collaborative study conducted by four laboratories, cervical cytology specimens from 830 patients were tested for the presence of HPV DNA using the ViraPap and Southern blot technique. The ViraPap was shown to have a sensitivity of 94.5% and a specificity of 95.5% when compared to Southern blot analysis (89). Intra and inter-laboratory reproduceability using a control panel prepared from the Hela cell line were 98.4% and 98% respectively.

The ViraPap kit uses probes complementary to the DNA of HPV types 6,11,16,18,31,33, and 35. The advantage of using RNA rather than DNA probes lies in the greater stability of the RNA-DNA complex (when compared to a DNA-DNA complex), thus allowing the use of more stringent conditions. However, since the kit uses high stringency conditions, only these specific types will result in a positive test result.

Comparison of Different Hybridization Techniques

It is important to understand that not only do these various hybridization tests have different sensitivities in the detection of histologically or cytologically documented disease, they also differ tremendously in their ability of detect HPV DNA in normal specimens with no evidence of disease. In addition, a given test method may have different sensitivities when applied to lesions of different grades.

A study by Caussy et al. compared Southern blotting, filter in situ hybridization, and in situ hybridization for their sensitivity and specificity in the detection of HPV DNA in condylomas, cancers, and normal cervical specimens (19). Sampling of the normal and cancerous cervices was not colposcopically directed, but was obtained from gross hysterectomy specimens. The diagnoses of condyloma and cervical cancer were confirmed histologically, as was the absence of lesions in the group of normal cervical specimens. It is of note that of the 33 normal cervical specimens 9 were found to have cervicitis, and 11 had metaplasia. The remaining 13 specimens were unremarkable. There was no evidence of CIN or HPV infection in any of these 33 specimens.

Probes capable of detecting HPV DNA of types 6,11,16,and 18 were used in this study. When the condylomas were assayed

by the three hybridization techniques described above, HPV DNA was detected in 82% by Southern blot, 62% by filter in situ, and 72% by in situ testing. When cancerous specimens were tested, HPV DNA was detected in 70%, 89%, and 30% of samples by Southern blot, filter in situ, and in situ hybridization respectively. Specimens which showed no histologic evidence of neoplasia or condylomatous changes were 13% HPV DNA-positive by Southern blot, 9.5% positive by filter in situ, and 6% positive by in situ. Condylomas and cancerous lesions were considered together for the purposes of calculating the sensitivities and specificities of disease detection. The Southern blot method had a sensitivity of 76%, and a specificity of 87%. The filter in situ technique had a sensitivity of 72% and a specificity of 90%, and the in situ technique, a sensitivity of 50% and a specificity of 93%. The sensitivity of the in situ method varied significantly between disease categories, with a sensitivity of 72% for condylomas, and only 30% for invasive cancer specimens.

Finally, the filter in situ and in situ techniques were compared to the Southern blot method as a standard. This comparison yielded sensitivities of 66 and 61% and specificities of 88 and 86% for the filter in situ and in situ hybridization techniques respectively.

The authors carried out experiments to determine the

lower limits of detection for each method. They concluded that the Southern method was capable of detecting 0.2 pg. of HPV DNA (0.2-0.3 copies per cell). The filter in situ method was capable od detecting as little as 0.1 pg. of HPV DNA. It was difficult to establish a lower limit in the in situ method, although, the authors were unable to detect Siha cells which contain 10 copies of HPV DNA per cell. They were able to detect Caski cells, which contain 500 copies per cell.

Clearly, any attempts at establishing the prevalence of HPV infection in the general population (with and without evidence of disease), will be greatly confounded by the variability in testing techniques. Conservative estimates of the prevalence of HPV infection in the sexually active population between the ages of 15 and 49, propose a figure of 10% (11). In a large population based study of cervical smears collected from 9,295 women attending gynecology clinics for routine screening, 8,755 (94.2%) of the smears were cytologically normal. 196 (2.1%) of the smears showed koilocytosis; 162 (1.7%) CIN1/2, 120(1.3%) CIN3, and 62 (0.7%) invasive cervical cancer. This study employed the filter in situ method to detect HPV DNA of types 6,11,16 and 18 in samples of exfoliated cells obtained at the same time as the cervical smear. HPV was detected in 9% of the cytologically normal smears; when CIN and invasive carcinoma were considered together, 40% contained HPV DNA (18). Only 30% of those

initially HPV--positive were detected by a follow up test 3 months later, and a number of patients whose specimens were initially negative were found to have HPV DNA on follow up. In view of this finding, and the 40% detection rate amongst invasive cancers using the filter in situ method, the authors propose that the prevalence rate is likely to be 2-3 fold greater than that detected by their study.

The Polymerase Chain Reaction

The polymerase chain reaction is a recently developed method of nucleic acid amplification which permits the detection of extremely small quantities of target DNA. Since its description in 1985, this method has been applied to a wide range of pathogens ranging from chlamydia to HIV.

The polymerase chain reaction requires a knowledge of the nucleotide sequence being amplified. A region of particular interest within a genome is selected for amplification. In the case of HPV this would most likely be a region which is highly conserved between different types, as this would give the widest range of detection. Once the target nucleotide sequence has been selected, stretches of nucleotides are identified, 25 to 30 base pairs long, and flanking the target region at the 5' and 3' ends. These flanking oligonucleotides are known as amplification primers and may be synthesized

using an automated DNA synthesizer.

A sample presumed to contain the target DNA is mixed with the amplification primers, free nucleotides (A,T,G, and C), and a thermostable DNA polymerase (such as TaqI). The mixture is subjected to a series of thermal cyclings. During each cycle the target DNA is denatured, the amplification primers anneal to their complementary sites, and the DNA polymerase extends the strand toward the other primers. After roughly 30 cycles the target DNA concentration has increased exponentially, over 1 million fold. This so-called "PCR product" consists of approximately 1 microgram of DNA, and can be analyzed by dot blot or Southern blot techniques, or even run on a gel and stained with ethidium bromide.

Theoretically primers can be designed which are type-specific, although this may be difficult within highly conserved regions. More typing information can be attained by a restriction enzyme digestion or by transferring the PCR product to nitrocellulose paper and hybridizing with type-specific probes. It has not yet been determined how many primer sets can be used simultaneously.

There are a number of advantages to the use of this amplification technique. It is highly sensitive, minute quantities of DNA can be used as starting material, and the

reaction can be carried out on paraffin-embedded specimens without DNA extraction. This provides a good means of retrospective analysis. The procedure is relatively easy to perform, (although preparation can be labor intensive), and lends itself to possible automation.

One disadvantage of the PCR is that it necessitates a knowledge of the genome being investigated. Whether new virus types or subtypes will be identified by this method remains to be seen. The clinical significance of the presence of viral DNA at a level only detectible following PCR has not been established. Finally, contamination of specimens or reagents with PCR amplification fragments can result in false positive results. Given the high sensitivity of this procedure, a single target fragment contaminating an otherwise negative specimen could easily produce a discernable amplification product. Contamination could also occur between specimens before processing, with a similar consequence. As a result of these contamination risks it has become crucial to run a large number of negative controls with each specimen being tested, and to take great care to avoid the dispersion of fragments in the laboratory by containment under a hood or use of a separate room.

A number of studies have investigated the presence of HPV in clinical specimens using this reaction. Of particular

interest are recent estimates of HPV prevalence based on detection following the PCR. A study by Tidy et al. used the polymerase chain reaction to look for HPV16 infection women with normal cytology, "dyskaryotic" smears, or invasive cervical carcinoma (29). The authors designed two sets of primers, A and B, which flanked a region in the upstream regulatory region, and C and D which recognized a segment within the E6E7 open reading frames. Each experiment included three controls, a positive control consisting of a known HPV16-positive cervical carcinoma, a negative control of DNA from the human cell lymphoblastoid line BJAB (HPV-negative), and water (to check for contamination). One hundred and forty normal cervical samples were tested with primers A and B; 84% of these specimens contained HPV DNA. Sixty-seven percent of the "dyskaryotic" smears contained HPV DNA, and 100% of the carcinomas yielded a positive amplification product. Twenty patients who had undergone laser treatment for CIN in the past and who were without evidence of disease at follow-up all showed a positive amplification product with the primers for HPV16. Primers C and D were used to confirm the findings in 22 samples. All the samples considered negative with primers A and B were also negative with the second primer set. Five of the 18 specimens positive with primers A and B were negative on testing with C and D. The authors attributed the failure to detect HPV DNA in these five specimens to the lower sensitivity of a longer amplification product. The

amplification product produced with each set of primers was of the appropriate size as determined by gel electrophoresis, and gave the appropriate pattern when cut with a restriction enzyme.

This study proposes a prevalence rate of HPV infection in the cytologically normal population far greater than that predicted using other detection methods. In addition it reports a greater rate of detection of HPV16 DNA in cervical carcinomas than has previously been described. Given the limitations of the PCR method discussed above, a study such as this one should be interpreted with caution. The primer sets used in this study flanked stretches within highly conserved regions of the HPV genome. Since there were no controls using samples containing HPV of different types, there is no assurance that these primers aren't amplifying a variety of different types of HPV, not simply type 16. Even so, the detection of HPV DNA of any type in such a large number of specimens from patients with normal cytology is an unprecedented finding. It is possible that HPV DNA does in fact exist in such a large percentage of the "normal" population, and has gone undetected because of the lower sensitivity of previously applied techniques. If HPV DNA is present in such a large proportion of the population should the understanding of the association between HPV and cervical cancer be revised? The clinical significance of detection of

HPV DNA by PCR is not at all clear. How much DNA should constitute an infection; is HPV a normal flora in some sense? It is also possible that the high rate of detection in this study reflects specimen contamination. Confirmation with a second primer set cannot allay fears that contamination occurred between specimens prior to PCR. The five specimens which were PCR positive by primers A and B only, may illustrate contamination with specific PCR fragments (fragments from the amplification with A and B, but not C and D).

A recent study analyzing the 210 smears which had served as the basis for the study described above found that the majority (63%) of women with normal smears were infected with type 16b, a previously unidentified subtype (15). This discovery was based on a slight difference in the molecular weight of the amplification products observed amongst the specimens following PCR. When the authors sequenced each of these products a new subtype was identified, differing from type 16 by a 21 bp deletion and 3 point mutations within the URR target being used for amplification. The authors found that less than 1% of the women with normal smears were infected with type 16a, and that dual infection with both 16a and 16b occurred in 20% of the normal specimens. Amongst the dyskaryotic smears, 43% had type 16a only, 20% were infected with type 16b only, and 7% showed dual infection. One hundred

percent of the carcinomas (22) contained type 16a alone.

The authors propose that the deletion in type 16b. leads to the loss of the consensus E2 binding site with subsequent reduction in the transcription of E6 and E7, and altered oncogenicity.

Both these studies serve to demonstrate the need for caution in interpreting the results of any new detection technique. Nonetheless, the PCR may allow for new methods of detection and may enlarge our understanding of disease transmission. An example of one such study is that done by Melchers et al., in which urine specimens were obtained from 17 male patients known to have condyloma acuminata in the meatus urethrae (21). Urine was also collected from 14 male volunteers with no evidence of HPV related disease. After the urine was centrifuged, a DNA extraction was performed on the sediment, and was found to contain between 100 and 500 ng. of chromosomal DNA by agarose gel electrophoresis. The polymerase chain reaction was carried out with primers flanking portions of the type 6 and type 11 genomes, and subsequently analyzed with agarose gel electrophoresis and Southern blot hybridization. HPV DNA of types 6 and/or 11 could be detected in 88% of the patients with condylomata acuminatum, but in none of the specimens from the control group. The authors conclude that urine specimens might provide

a means of screening for HPV infection, although such a method may only detect those men with infection in the urinary tract, and may miss those individuals with lesions of the squamous epithelium of the penis. The finding of HPV DNA in specimens presumably containing cells exfoliated from the genito-urinary tract also supports the notion that HPV could be transmitted to women in the semen of infected males.

MATERIALS AND METHODS

Subjects

The participants in this study were forty women who were referred to the Yale Gynecologic Oncology Clinic for evaluation of an abnormal Pap smear. The women ranged in age from 18 to 64 with an mean age of 30. Seven of the 40 women were pregnant at the time the study specimen was collected.

The cytologic abnormalities that prompted referral to the Yale Oncology Clinic ranged in severity from "some features suggestive of condylomatous changes," to "carcinoma in situ." In most cases the study specimen was obtained at the time of the subject's initial evaluation at Yale; in a few instances the subject was already being followed at the Oncology Clinic, and the sample was collected during a return visit. Both initial and follow-up evaluation at the Oncology Clinic consisted of a pertinent history, and physical examination including a Pap smear, colposcopy, and endocervical curettage and colposcopically directed cervical biopsies whenever appropriate. The investigational use of the diagnostic test was explained to each subject, and with her consent an endocervical cytology specimen was obtained.

Specimen Collection

Specimens were collected by Obstetrician-Gynecologists at the Yale Gynecologic Oncology Clinic using the ViraPap Specimen Collection kit. After visualization of the cervix and collection of a Pap smear using a cotton tipped swab and Ayre spatula, a dacron tipped swab was used to sample exfoliated cells from the endocervical canal. Immediately after sampling the swab was placed in 1 milliliter (ml.) of transport medium containing a cheiotropic agent and 0.05% w/v Sodium Azide to retard bacterial growth. Specimens were stored at room temperature for no more than one week before being frozen at -20 degrees Celsius for up to two months.

In most cases colposcopy was performed immediately after ViraPap specimen collection. Any colposcopic findings (aceto-white change, abnormal vascularity, punctation, mosaicism, visible condylomata) were noted, and colposcopically directed cervical biopsies and endocervical curettage were carried out when appropriate. Cervical biopsies and endocervical curettage specimens were transported to the Department of Pathology in a 10% Formalin solution, prepared for light microscopy with Hematoxylin and Eosin staining, and read by pathologists in the Yale department of Pathology. Pap smears were screened by certified cytotechnologists and those with abnormal findings were referred to department pathologists for

interpretation.

After approximately fifty specimens were collected, frozen samples were thawed and assayed. A technical service representative from Life Technologies supervised the processing of the specimens. In instances where the clinician had substituted a wood-shafted swab for the plastic-shafted swab provided in the manufactured collection kit, the specimen was discarded because of the possibility of a false negative result after wooden material has been stored in the transport media. Specimens which were not accompanied by a concurrently taken Pap smear were also disqualified from the study. In all, 40 specimens were deemed acceptable. Each specimen was numbered, and the date of collection, clinician attending the patient, as well as the subject's name, unit number and date of birth were recorded on a tally sheet. Special note was taken of the specimen appearance, and yellow, dark yellow or visibly bloody specimens were identified.

ViraPap: HPV DNA Detection by Hybridization Techniques

Specimens were prepared for hybridization according to the instructions provided in the ViraPap Human Papillomavirus DNA Detection Kit, and utilizing reagents provided in the same self-contained kit. The kit contains positive controls made up of disrupted Hela cells in ViraPap transport medium. Hela

cells are a transformed human cell line derived from a cervical carcinoma containing integrated copies of the HPV-18 genome. The high positive control contains 2.0 ml. of 1.0 x 10^5 cells/ml., and the low positive control a higher dilution at 1.0 x 10^4 cells/ml. A negative control consists of 1.0 ml of 5 x 10^5 cells/ml. disrupted HTB-31 cells, a transformed human cell line derived from a cervical carcinoma which does not contain any HPV sequences. All three controls were processed in the same manner as the patient specimens.

Two drops of blue "Sample Preparation Reagent" were added to each specimen tube. This reagent is a bacterial protease which serves primarily to disrupt the integrity of the endocervical cells in solution and release the DNA into the medium. Each tube was recapped and vortexed and then incubated at 37 degrees Celsius for 1 hour. The samples were vortexed again following incubation and the dacron swabs removed after care was taken to express as much of the medium as possible. Two hundred and fifty microliters (ul.) of the sample was removed and placed in a 12 x 75 millimeter (mm) test tube and 750 ul. of "Sample Diluent," a dilute base solution, was added and the specimens were incubated at room temperature for five minutes. This basic solution effectively denatures any DNA present. Each 1.0 ml specimen was then placed in a well in the ViraPap Filter Manifold, and filtered through a presoaked nylon membrane with a vacuum pressure of

13mm Hg.

The nylon membrane was then incubated at 60 degrees Celsius with a prehybridization reagent containing formamide, blocking reagents, stabilizers, and a kinetic enhancer. After a 30 minute incubation the membrane was blotted dry and the "Hybridization Reagent" was added. The hybridization reagent contains 5 microcurie of ^{32}P labelled RNA probes complementary to the DNA of HPV types 6, 11, 16, 18, 31, 33, and 35. The membrane was then allowed to incubate at 60 degrees for two hours.

When the hybridization incubation was complete the membrane was washed with two high stringency buffers and treated with an RNAse reagent to reduce nonspecific binding of the RNA probes. Wash #1 was a 0.26 M phosphate buffered saline solution containing 0.026 M EDTA and 4.8 M NaCl. The membrane was covered with 50 ml. of buffer and incubated at room temperature with gentle agitation for three minutes. These washes were carried out a total of three times. RNase reagent was then added to cover the membrane and allowed to incubate with shaking for 15 minutes at 37 degrees Celsius. One hundred and fifty ml. of Wash #2, a 100 mM phosphate buffered saline solution with 10 mM EDTA and 1% w/v SDS, was added and the membrane was incubated with shaking for 5 minutes at 60 degrees. This wash was also carried out a total

of three times. When the washing procedure was completed the membrane was blotted dry and placed in a film cassette adjacent to Kodak type 57 film with intensifying screens, and allowed to expose at -70 degrees. The film was developed after 7 days (Figure 1).

ViraType: Identification of three distinct viral groups

The protocol accompanying the ViraType Human Papillomavirus DNA Typing Kit was followed during the typing procedure. Three type specific controls were provided in this kit. The controls were prepared from HPV DNA added to inactivated HTB-31 cells in transport media. One positive typing control contained HPV DNA of type 6 and 11, the second, HPV DNA of types 16 and 18, and a third, HPV DNA of types 31, 33, and 35. A negative control contained only inactivated HTB-31 cells in transport media.

Only those specimens which had yielded a positive ViraPap test were passed through the typing procedure, with the exception of two specimens which served as internal negative controls (Specimens #8 and #22). There were approximately 750 ul. of the original specimen remaining after the protease treatment step carried out in the ViraPap protocol. Four hundred and fifty ul. of this specimen was transferred to a clean 12x 75 mm test tube. Two milliliters of Sample Diluent

(a chemical denaturing reagent) was added to each specimen, mixed thoroughly and allowed to incubate at room temperature for 15 minutes. Three separate nylon membranes were prepared for filtration and labelled 6/11, 16/18, and 31/33/35 respectively. Each membrane was subsequently placed in the ViraPap Filtration Manifold and 800 ul. aliquots of each specimen were placed in corresponding wells on each membrane. The three positive controls and the negative control were applied to each of the three filters as well. After an aliquot of each sample had been loaded in the filter manifold, vacuum pressure was applied to 13 mm Hg. The manifold was washed well with soap and water after each filtering.

Prehybridization was carried out using a formamide solution containing blocking agents and a kinetic enhancer. The three filters were incubated at 60 degrees for 30 minutes, and then blotted dry. There were three separate hybridization mixtures, each containing viral group-specific probes. The RNA probes in the ViraType kit were prepared by in vitro transcription of recombinant plasmid which each contain essentially the entire genome of a particular viral type. ^{32}P labelled RNA sequences complementary to types 6 and 11 were added to one hybridization reagent, RNA complementary to types 16 and 18 were added to a second, and RNA complementary to types 31, 33, and 35 to a third hybridization mixture. A separate membrane was incubated with each of the

hybridization reagents for two hours at 60 degrees Celsius. After the hybridization incubation the membranes were washed with the same high stringency washes used in the ViraPap kit, and likewise treated with an RNase reagent to minimize nonspecific probe binding. The schedule and sequence of steps were identical to those used in the ViraPap protocol. The membranes were then placed in a film cassette with Kodak type 57 film and intensifying screens and exposed at -70 degrees Celsius. The film was developed after 7 days (Figure 2).

Chart Review

A chart review was made of all the gynecologic oncology records of participating subjects. The objectives were to ascertain gravity and parity, and most importantly, to document the colposcopic findings at the time of specimen collection and to review the final pathology reports concerning any concurrently taken cytology and pathology specimens.

Pap smears taken at the time of ViraPap specimen collection were scored as positive (indicative of disease) if the cytologic interpretation included "atypia," "viral atypia," koilocytosis, "condylomatous changes," or any grade of CIN. Cervical biopsy and endocervical curettage specimens were scored similarly. In instances where the pathologist read

the cytology or histology as representing a range of pathology (ie. CIN1-CIN2), the more severe grade was recorded. Likewise, when multiple biopsies taken from a single patient yielded varying degrees of pathology, the most severe grade was recorded. Smears and biopsies showing "inflammatory atypia" were not scored as positive (see Appendix).

Polymerase Chain Reaction

Using the Wisc-5 computer program (Version 5, Sequence Management Software), the entire genomes of type 16 and 18 were aligned and staggered so as to provide for the greatest degree of homology. The regions containing the E6 and E7 open reading frames (ORF's) were outlined on each genome and an effort was made to select stretches of nucleotides within each of the type 16 open reading frames that had little if any homology to the corresponding regions of type 18. Four oligonucleotides were chosen, each between 23 and 25 base pairs in length, and subsequently named HPV16-1, HPV16-2, HPV16-3, and HPV16-4. HPV16-1 and 2 lay within the type 16 E6 ORF, and framed a sequence of nucleotides 183 base pairs long (including the length of the primers themselves). HPV16-3 and 4 lay within the type 16 E7 ORF and flanked a sequence 163 base pairs long. The oligonucleotide primers were synthesized on a DNA synthesizer with Trityl on, and purified by oligopurification cartridge (Figure 3a).

The annealing temperatures of the primers were approximated based on the G, T, A, and C content of each oligonucleotide, and the PCR machine was programmed to cycle with an annealing temperature of 55 degrees Celsius. These conditions were thought to be stringent enough to minimize nonspecific binding, while still allowing for effective elongation. The Polymerase chain reaction was carried out using a protocol modified from the original Elmer Cetus form. A "master mix" was prepared containing all the components necessary for a DNA synthesis reaction except the DNA template itself: free nucleotides (Promega), Taq polymerase (a thermostable DNA polymerase, manufactured by Perkin Elmer Cetus), oligonucleotide primers flanking the region to be amplified (manufactured as described above), a detergent (Tween), 10x Buffer with a magnesium concentration of 15 mM, and water. In order to test the viability of the HPV primers before applying them to patient specimens, the polymerase chain reaction was first run with serial dilutions of pBR322 plasmid containing HPV-16, kindly provided by Dan DiMaio's laboratory. A 1:10 serial dilution series was made of pBR322/HPV-16 plasmid (1.065 micrograms/ microliter) ranging from 1.0×10^{-2} to 1.0×10^{-8}. 5 microl. of each plasmid dilution and 45 ul. of "pcr master mix" were added to a 1.5 ml. sterile siliconized Eppendorf tube. A drop of mineral oil was placed on top of each reaction mixture to minimize

evaporation during the thermal cycling, and the tubes were placed in a Perkins Elmer Cetus DNA Thermal Cycler. The samples were passed thirty times through a cycle of successive incubation steps: denaturation at 94 degrees Celsius for 45 seconds, annealing at 55 degrees for 45 seconds, and extension at 72 degrees for two minutes. After the cycling was complete, 5 microl. of the reaction mixture was drawn up and run on a 4% agarose gel with a KB ladder serving as a standard. The gel was then stained in ethidium bromide for 45 minutes (Figure 3b).

DNA Extraction of Remaining ViraPap Specimens

Once the assay was optimized, we applied the technique to patient specimens. To provide an accessible, concentrated DNA template, a DNA extraction was carried out on the remaining aliquot of the original ViraPap specimen. A protocol for extracting sample DNA from transport medium for Southern blot analysis was provided by Life Technologies, Inc. This protocol was followed with minor modifications.

A 250 ul. aliquot of each of the original cytology specimens (or as much as was remaining) was transferred to a 1.5 ml. Eppendorf tube. Kit positive controls were also processed in the same manner. If less than 250 ul. of specimen was available, the sample was augmented with ViraPap transport

medium. An equal volume of phenol/chloroform/isoamyl alcohol (water-saturated and neutralized) was added and the mixture vortexed for 10 seconds. The specimens were then centrifuged in a microfuge (12,500 x g) at room temperature, and the resultant aqueous upper phase transferred to a clean Eppendorf. The lower phenol/chloroform phase was reextracted with 250 ul. of TE (10 mM This-HCl, pH 7.5, 1 mM EDTA), vortexed, centrifuged, and the upper aqueous phase pooled with the previously separated phase. 500 ul. of chloroform/isoamyl alcohol (49:1) was added to these pooled phases, and the mixture vortexed, then centrifuged for 1 minute, 12,500 x g., at room temperature. The resultant upper aqueous phase was transferred to a clean Eppendorf, and 50 ul. of 2.75 M NaAc and 600 ul. of isopropanol were added. This mixture was vortexed, and then centrifuged for 30 minutes in a microfuge (12,500 x g) at 4 degrees Celsius. When the centrifuging was complete, the supernatant was drawn off, and the tube contents were allowed to dry overnight at 37 degrees. In most cases there was a visible DNA pellet. The pellet was then resuspended in 50 ul. of TE. Five microliters of each specimen were passed through the PCR with primers HPV16-1, 2, 3, and 4; producing amplification products corresponding to the E6, E7 and E6E7 targets. Figure 4 shows the results of the E6 amplifications.

Restriction Digest on Specimens Identified as 16/18 Positive by Either ViraType or PCR

The type 16 and type 18 genomes were entered into the Wisc-5 program and a map was produced indicating sites of restriction enzyme cutting. The regions corresponding to the E6 amplification product were examined within each genome; for each type,one enzyme was selected which cleaved the DNA within that region, but which had no activity in the alternate type. Taq I (manufactured by Bethesda Research Laboratories [BRL]) was selected as the type-16 specific enzyme; Taq I recognizes a site within the type-16 DNA sequence corresponding to the E6 amplification product which would be expected to yield two fragments of 134 and 49 nucleotides. Hae III (BRL) was chosen as the type 18 specific enzyme; this enzyme would be expected to produce two fragments 139 and 63 nucleotides long.

All the specimens ViraType-positive for type 16/18, as well as any specimens which had produced a discernable E6 amplification product by PCR were included in this experiment. Also subjected to restriction digest were all three positive ViraType controls, a 10^{-4} dilution of pBR322-HPV-16 plasmid, a negative control (master mix), and one 31/33/35-positive and one 6/11 positive specimen. Using the PCR with primers HPV16-1 and HPV16-2, new E6 amplification product was made from each specimen in preparation for the restriction digest

experiments. Five microliters of the resultant products were run on a 4% agarose gel for one hour at 100 volts, and the DNA stained with ethidium bromide. The specimens could be easily grouped into those which produced a bright, easily discernible signal, and those with a fainter band at the appropriate position. In the subsequent digest, 5 ul. of the former specimens were entered into the digestion, while 10 ul. of the latter specimens were used.

5 or 10 ul. of PCR product, 1 ul. of TaqI, 2 ul. of buffer (React 2), and water were mixed in a .5 ml. Eppendorf to bring the volume of the reaction mixture to 13 ul. The samples were allowed to incubate overnight in a 37 degree water bath. The entire specimen was then run on a 4% agarose gel at 100 volts for approximately two hours and stained with ethidium bromide (figure 5a).

This procedure was then repeated using Hae III, the type-18 specific enzyme (figure 5b).

Determination of Specimen Adequacy

We developed a random primed genome probe for human DNA with the intention of determining whether human DNA was present in all the samples. Because of their easy accessibility, white blood cells were selected as a source of

DNA for creating a probe as well as a target with which to test probe sensitivity.

The target DNA consisted of known dilutions of white blood cells processed in a manner mimicking the ViraPap protocol for endocervical specimens. The buffy coat was isolated from 1 and 1/2 ml. of whole blood with a (Ficoll-Paque and PBS gradient). The white blood cells were then washed with five volumes of PBS and centrifuged at 7 x g. for five minutes. This wash was repeated, and the final supernatant was decanted, leaving 1 and 1/2 ml. A 100 ul. aliquot of the white cell solution was then read in a Ortho Diagnostic Laser cell counter; the white cell number was calculated to be 0.9 cells/ul. The solution was centrifuged, and the pellet was then resuspended in 1.5 ml. of the ViraPap transport medium. Two drops of the Sample Preparation Reagent were added and a serial dilution was made corresponding to 2.25×10^5, 1.125×10^5, 1.125×10^4, 1.125×10^3, 1.125×10^2, and 1.125×10^1 cells. Each dilution, in a 250 ul. aliquot, was then applied to a nylon membrane using the ViraPap Filter Manifold in the same manner used in loading patient specimens. After filtration the membrane was baked at 55 degrees for 5 minutes and then exposed to ultraviolet (302 nanometer wavelength) for five minutes to cross link any unbound DNA.

In preparing the probe, white blood cells were isolated

from whole blood by the same method described above, and labeled by incorporating ^{32}P labeled adenosine triphosphate using denatured WBC DNA as a template. The labeling was carried out using the Primagene^{TM} labeling system, which employs a mixture of random hexanucleotides to prime DNA synthesis in vitro.

The isolated white cells in solution were centrifuged, and the pellet was resuspended in 0.45 ml.Proteinase K Digest with 50 ul. of Proteinase. The mixture was incubated at 55 degrees for 1 and 1/2 hours to assure digestion, and then a DNA extraction was performed using the Chloroform/Phenol method described earlier for DNA extraction from ViraPap cytology specimens. The WBC DNA product was washed with ethanol, dried overnight, and resuspended in 25 ul. of TE. A calculation of DNA content was made using spectrophotometer measurements, and a volume estimated to contain 25 nanograms (ng.) of DNA was entered into the Primagene assay.

25 ng. of WBC DNA dissolved in TE (1x) were denatured by heating at 95 degrees for 2 minutes. The sample was immediately placed on ice to prevent reannealing, and the Primagene reaction mixture was assembled. The components of the Primagene system include a "labeling buffer" which contains a mixture of random hexanucleotides which will serve as primers for DNA synthesis, a mixture of unlabeled

nucleotides, A, T, G, and C, and Klenow enzyme, a DNA polymerase. ^{32}P labeled Adenosine was chosen as the labeled nucleotide (50 microcuries, 3000 curies/mmole). 10 ul. of labeling buffer, 2 ul. of nonlabelling dNTPs, 1 ul. of denatured WBC DNA template, 2 ul. of nuclease-free BSA, 5 ul. of ^{32}P-dATP, 5 units of Klenow enzyme, and 25 ul.of sterile water were added to a microcentrifuge tube, mixed gently, and allowed to incubate at room temperature for 1 hour. The reaction was terminated by heating at 95 degrees for 2 minutes and subsequent chilling. EDTA was added to 20 mM, and the probe was stored at -20 degrees.

The probe was initially tested against the membrane prepared with known dilutions of whole WBCs. The membrane was placed in a "seal-a-meal" bag with 15 ml of prehybridizing solution containing salmon sperm DNA, and incubated overnight in a 55 degree waterbath. 5 ul. (5 microcuries) of ^{32}P-dATP were added, and the solution was allowed to hybridize for 48 hours. The hybridization solution was decanted and the membrane was washed twice with a low stringency buffer (Wash #1, 2 x SSC/0.1% SDS), at room temperature, for 10 minutes. The membrane was then washed with a slightly more stringent buffer (Wash #2, 1 x SSC/0.1% SDS) at room temperature for 30 minutes. The membrane was blotted dry and placed in a film cassette with Kodak film and intensifying screens, and was allowed to expose for 7 days (figure 6).

The ViraPap membranes were stripped of previous RNA probes by a shaking incubation at 90 degrees with a high stringency wash (50% Formamide). The membranes were subsequently washed twice with a low stringency wash for 10 minutes at room temperature. Prehybridization of the membranes was carried out overnight at 55 degrees, and 5 ul. of ^{32}P labeled probe were added to the solution in the morning. After a 24 hour incubation, the membranes were washed with Wash #1 and Wash #2 as described above, and the membrane was placed in a film cassette with intensifying screens. The film was allowed to expose for 6 hours at -70 degrees (figure 7).

RESULTS

Chart Review

Forty gynecologic oncology charts were reviewed in this portion of the study. Parity at the time of specimen collection was reviewed, and 18 of 40 (45%) of the subjects were found to be nulliparous, 14 of 40 (35%) were parous, 7 (17%) were pregnant. For 1 subject there was no parity documented.

Twenty-one of the 40 cervical smears (Pap smears) taken at the time of ViraPap specimen collection were noted to have abnormal findings (53%). Of these, 10 were described at showing atypia or "koilocytic atypia," and 11 smears were read as displaying some degree of dysplasia or CIN 1, 2, or 3. The remaining 19 Pap smears were read as normal.

Of those subjects who had abnormal findings on pap smears, 11 (55%) had positive ViraPap tests. There were 5 cases in which a normal pap smear was accompanied by a positive ViraPap test. In all of these cases there was evidence of disease by other parameters (cervical biopsy or colposcopy.)

When cervical biopsy pathology was examined, there were

19 patients with evidence of disease ranging from koilocytic atypia to invasive Ca. The ViraPap test detected HPV DNA in the endocervical swab specimens of 9 (47%) of these cases. There were 10 cases of cervical pathology for which the ViraPap test was negative. There were, however, 9 subjects in whom cervical biopsy was not performed despite abnormal findings on colposcopy. The ViraPap detected HPV DNA in 4 of these cases, 1 of which had a normal accompanying Pap smear. Interestingly, there was one case in which a positive ViraPap test was obtained despite a normal cervical specimen: this patient had also had a vaginal biopsy performed, and this proved to be positive for dysplasia. Another subject had both negative ViraPap and cervical biopsy results, but had a vaginal biopsy which showed koilocytic atypia. Both these subjects had normal Pap smears.

With one exception, all the patients in the study had a Pap smear taken at the time of ViraPap specimen collection. The exception was a patient who was evaluated at the gynecologic oncology clinic, with simultaneous referral to the Pathology department of previously taken biopsy specimens showing invasive cancer. This patient had a nodular, cancerous cervix visible on gross inspection, and no Pap smear was taken. For the purposes of this study, this patient was scored as having had a positive Pap smear because her cervical disease would have been evident to any clinician taking a Pap

smear.

In this study, cervical biopsies were not obtained in a number of instances in which there were colposcopic findings suggestive of disease. There were two reasons biopsy was not carried out; first, several of the patients were pregnant, and cervical biopsy was thought to pose an unnecessary risk, and second, a number of patients had had previous colposcopic and histologic evaluation before presenting to the YNHH gynecologic oncology clinic, and had their pathology specimens referred and reread by the Yale Pathology department. When these specimens showed evidence of disease, it was sometimes not necessary to repeat biopsies as it was not felt that the information provided would alter management. Because these biopsies were not obtained at the same time as the rest of the material analyzed in this study, the findings were not included in our data.

ViraPap Results

Any specimen whose signal was equal to or of greater intensity than the signal corresponding to the kit low positive control was considered to be positive. A positive result indicates the presence of HPV DNA of at least one of types 6, 11, 16, 18, 31, 33, and 35. ViraPap kit protocol recommends that a specimen which yields a signal which is more

distinct than the negative control, but less than the low positive control should be considered "borderline" and that another specimen be obtained from that patient, and the assay repeated.

Sixteen of 40 specimens (40%) tested positive for the presence of HPV DNA of types 6, 11, 16, 18, 31, 33, or 35. Many of the autoradiograph signals produced by the samples were of far greater intensity than even the high positive controls, and there was virtually no background. (note: The signal produced at position A3, corresponding to specimen #8, was thought to represent a rim of HPV DNA-positive fluid drawn over from the adjacent, highly positive specimen (#13). This specimen was not counted among the positives, and was subsequently established to be negative.)

The ViraPap protocol includes a disclaimer with regard to bloody specimens, stating that "blood may cause false negative results." Six specimens among the total 40 had been noted to be dark yellow or brown at the time of ViraPap assay, suggesting the presence of blood in the solution. Only one of these, #40, resulted in a positive ViraPap test (figure 1).

ViraType Results

Each of the three membranes represented a different viral

group typing. Any signal generated by a patient specimen which was equal to or less intense than the kit negative control was interpreted as negative; a relative absence of HPV DNA of the type being probed for on that particular membrane. A signal of greater intensity than the negative control was scored as positive. If a patient specimen produced a signal greater than the negative control on more than one membrane, the possibility of infection with multiple types of HPV was considered, and interpreted in light of the potential cross reactivity described below.

Interpretation of the ViraType autoradiograph was a straightforward process confounded only by slight cross reactivity between the positive controls for types 16/18 and 31/33/35. A region of homology between type 16 and type 31 allows for some cross-reactivity between these probes when large amounts of HPV DNA are present. For this reason, a specimen strongly positive for HPV DNA of types 16/18 may show a signal on the 31/33/35 membrane as well. If the signal on this second membrane is of equal or lesser intensity than the 31/33/35 positive control on the 16/18 membrane, cross-reactivity, and not a true 31/33/35 positive should be suspected. It was not possible to determine whether more than one viral type within a viral group was present, as the ViraType probes were mixtures of different viral types.

In all, 21 specimens were assayed with the ViraType kit. Of these, 16 had previously tested positive with ViraPap. Of the 16 specimens, 1 was typed as 6/11; 6 were typed as 16/18; 6 were typed as 31/33/35; and 2 specimens (#17 and #28) appeared to have multiple infection with HPVs from groups 16/18 and 31/33/35, as these specimens had a strongly positive result on the 16/18 membrane, and also produced a signal on the 31/33/35 membrane of greater intensity than the corresponding control on the 16/18 filter. One specimen (#40), which had produced a positive signal by ViraPap, did not produce a positive signal on any one of the three viral-group membranes, and hence was interpreted as "untypeable". The additional 5 specimens assayed by ViraType included 2 used as internal negative controls (#22,25), a third questionable specimen (#8), and two specimens not previously run on ViraPap, (#45,and 46). All five of these additional specimens had a negative result by ViraType assay (figure 2).

PCR Results

The prime objective in carrying out the Polymerase Chain Reaction was to verify HPV type 16 positive specimens by a very sensitive means, and to identify any additional positives not detectible by the nucleic acid hybridization methods already used. The ViraType assay does not differentiate between types 16 and 18, so starting samples were known only

to be 16/18 positive.

When the polymerase chain reaction was carried out on the serial dilutions of pBR322-HPV16 plasmid, clearly defined bands were seen at the predicted molecular weights. Primers HPV16-1 and HPV16-4, which flank a stretch of genome including both E6 and E7, produced a 398 nucleotide amplification product from dilutions as high as 10 (-7). Primers HPV16-1 and HPV16-2 produced a 183 nucleotide amplification product with equal sensitivity. The E7 amplification product, flanked by HPV16-3 and HPV16-4 was visible from a starting dilution of 10 (-6). This last reaction series was of interest because of the formation of "primer-dimers" by oligonucleotide primers not consumed in the amplification reaction (Figure 3b).

The study samples were divided into two sets and subjected to the PCR. The first reaction series included four specimens which had tested negative by ViraPap and were not believed to contain any HPV DNA, the six specimens identified as type 16/18 by Viratype, the two specimens demonstrating coinfection, as well as one 6/11-positive specimen, and two 31/33/35 specimens. DNA extracted from the three kit controls for 6/11, 16/18, and 31/33/35 was also included.

All the specimens identified as type 16/18 by ViraType produced a discernable band at the position of the E6 product

when amplified using the HPV16-specific primer for E6. Neither the four specimens originally negative by ViraPap, nor the type 6/11 specimen produced a positive result. One of the 31/33/35-positive specimens (#44) gave a weakly positive signal, while the other did not. DNA extracted from the kit controls for type 16/18 gave a strongly positive band on the gel, and the kit control for type 6/11 produced a weakly positive result. The kit control for types 31/33/35 did not produce a visible signal.

The second reaction series was not known to include any specimens containing HPV 16/18 DNA. Four additional positive specimens were readily identifiable after PCR. Two of the four were strongly positive; one specimen (#40), had been previously categorized as "untypeable" following a positive ViraPap result and negative ViraType test, and the other had been negative by both ViraPap and ViraType testing. The remaining two positive specimens (#s 21,22) could be considered weakly positive, and produced bands with an intensity equal to or less than that of the 6/11 control on the first gel (Figure 4).

Results; Restriction Digest

Subjecting the specimens to the PCR identified several additional 16/18 positive specimens. While the oligonucleotide

primers used in the PCR were designed with type 16 specificity in mind, all the specimens identified as 16/18 positive by ViraType had also been successfully amplified by the PCR, so the primer set's ability to differentiate between type 16 and 18 by preferentially binding to type 16 sequences had not been demonstrated. A restriction digest with type-specific enzymes was undertaken to differentiate between type 16 and type 18 positive specimens as a means of ascertaining primer specificity.

Taq I cut all the 16/18 positive specimens (as identified by ViraType and /or PCR), producing visible fragments of approximately 140 nucleotides in length, as predicted by restriction mapping of HPV type 16. The specimens which had been weakly positive by PCR (17, 21, 22, 44, and C-1) were indistinguishable after digestion (Figure 5a).

Digestion with HAE III, the type 18 specific enzyme, did not result in any alteration in fragment size from uncut form. The PCR weak positives were still faintly visible at a position corresponding to their uncut molecular weight (Figure 5b).

Results; Sample Adequacy

The ViraPap kit uses probes which are capable of detecting the DNA of HPV types 6/11, 16/18, and 31/33/35 only. When a specimen is assayed and a negative result is obtained no inference can be made with regard to the possibility of infection with another type of HPV, nor can one be assured that an adequate specimen was initially obtained from the patient. Generally. 10-100 labelled copies will produce a autoradiographic signal. This number is thought to represent the lower limit of resolution by this technique.

Use of the human DNA probe on known dilutions of white blood cells demonstrated that the probe was capable of detecting as few as (1000) cells after a seven day exposure. When the probe was applied to the ViraPap membranes and allowed to expose for 6 hours, every patient specimen produced a signal darker than the high positive control, known to contain 2×10^5 disrupted Hela cells. The intensity of the signal produced by a given specimen with the human DNA probe did not correspond to the signal intensity of the same specimen when probed with either the ViraPap or ViraType probes (Figure 7).

DISCUSSION

Subjects

The patients in this study could all be considered to have a risk factor for the later development of cervical cancer on the basis of a previously abnormal Pap smear. Information concerning age at first coitus, number and health status of sexual partners, and histories of sexually transmitted diseases was not universally reported in the gynecologic oncology records, and hence could not be analyzed in a further consideration of risk status. A large proportion (17%) of the patients were pregnant; as discussed previously, pregnancy may be considered an altered state of immunity and may lead to a reactivation and increased rate of viral production. For these reasons, the patients in this study are not representative of the general population and the results of this study must be interpreted with reference to a "high risk" population.

While most of the oncology records recorded number of sexual partners, and it can be assumed that if a patient had not been sexually active, this fact would have been recorded as remarkable, there was not universal documentation of virginal/nonvirginal status among the study patients. In light of recent anecdotal reports of HPV transmission to the

genitals without sexual contact, future studies with a patient population referred for abnormal cervical cytology might investigate sexual activity.

ViraPap and the Pap Smear

Studies assessing a detection method must choose some standard definition of disease by which to measure the new test's sensitivity. Most investigations of hybridization methods for HPV DNA detection have used histopathologic evidence of disease as the standard for comparison. In this study, all abnormal findings on Pap smears, ranging from koilocytic atypia and other cytologic findings suggestive of HPV infection, to CIN3 were taken to represent "cervical disease". Such a broad classification system, while failing to distinguish between simple viral infection and CIN, is acceptable in this investigation because all the specimens thereby included warrant follow-up and further diagnostic interventions such as colposcopy and cervical biopsy.

Overall, cytologic methods (Pap smear) detected cervical disease with the greatest frequency. Twenty-one of the 40 Pap smears obtained on the day of study (52.5%) showed evidence of disease as defined above. It is of note that all (100%) of the subjects in this study were referred for an abnormal Pap smear in the recent past, thus highlighting the limitations

of relying on the Pap smear alone to demonstrate disease. The Pap smears with cytologic abnormalities were not necessarily those found to contain HPV DNA; 11/21 of the abnormal Pap smears (52%) were positive for the presence of HPV DNA.

The ViraPap detected HPV DNA in 16/40 (40%) of concurrently taken endocervical swabs. The specimens in which HPV DNA was detected were not necessarily those which showed cytologic abnormalities; Most significantly, 5/19 (26%) of the cytologically normal Pap smears were found to contain HPV DNA. These latter specimens represent five instances in which a woman with an increased risk of developing cervical cancer may have failed to be identified. Typing of the virus found in these five instances revealed four infections with the "high risk" group, types 16 and 18, and one infection with the group 31/33/35, which is associated with an intermediate risk of developing cervical neoplasia or a precursor lesion (20, 26, 27, 28, 64). When the four 16/18-positive specimens were subjected to PCR and restriction digestion, they were ascertained to be type 16. It is significant that these five cases all had evidence of disease by another more invasive parameter (cervical biopsy, or colposcopy). Colposcopy and biopsy are not performed in routine screening, so those patients with normal Pap smears may have been missed by ordinary screening methods, but were identified as harboring HPV DNA by the ViraPap test.

A single pathologist (S.F.) was asked to rescreen the 40 Pap smears obtained on the day of study; while he was aware of the purposes of this study, S.F. did not have any knowledge of the previous interpretation given the Pap smears, nor did he know of the ViraPap results for each patient. Because the rescreening was not carried out in a completely blind manner, calculations of interobserver variability would not be accurate. Nonetheless, an important finding emerged from the rescreening efforts; in the 5 cases in which a negative Pap smear had a corresponding positive ViraPap test, 4 of the Pap smears were described as inadequate on review. Two smears were described as unsatisfactory because of the absence of endocervical cells, one was of unacceptable quality as a result of airdrying, and one had obscuring purulence and cytolysis. The fifth smear, considered adequate, was read as CIN3 on rescreening--it had been interpreted as normal on initial evaluation. These findings serve to highlight the importance of specifying "sample adequacy" when reporting cytology interpretations, and also demonstrate the role that an HPV DNA detection test such as ViraPap could play in identifying patients in need of further evaluation.

A positive ViraPap was found to be a good predictor of the presence of cytologic abnormalities. In 11/16 (69%) of instances in which HPV DNA was detected, cytologic

abnormalities were present, ranging from atypia to CIN3. The ViraPap appeared more sensitive in the detection of dysplasias than in cases of atypical cytology. Six of 7 (86%) of patients with CIN 1, CIN2, or CIN3 had HPV DNA detected by ViraPap, while in only 4/13 (31%) of patients with atypia, or condylomatous changes, was HPV DNA detected. Because of the small number of specimens in each of these categories, actual sensitivities were not calculated.

There were 10 cases of abnormal Pap smears in which HPV DNA was not detected by ViraPap. In nine of these cases there was evidence of disease by another parameter, either tissue biopsy or colposcopy. In one case neither biopsy or colposcopy was performed for reasons which are not clear. These 10 specimens could be considered false negatives. If a false negative rate is calculated, taking as the numerator those cases in which disease is present but HPV DNA is not detected by ViraPap, and as the denominator, all cases in which disease is present as shown by cytologic examination, a value of 46.7% is obtained. This is slightly better than some of the estimates of false negativity rates for the detection of cervical disease by Pap smear (86). The factors contributing to the high rate of false negative Pap smears has been discussed in some detail (see section on the Pap smear). There are a number of reasons a ViraPap test could yield a false negative result. First, the ViraPap only contains HPV

RNA probes complementary to the DNA of types 6, 11, 16, 18, 31, 33, and 35, and the assay is run under highly stringent conditions. Any other types of HPV will not be recognized by the probes, and thus will be undetected in this test. Heretofore unidentified HPV types are unlikely to hybridize under the stringent conditions used, even with significant homology. Second, an extremely low level of infection with few copies of HPV DNA, may escape detection by this test. The low positive control provided in the kit contains 4 x 10 (4) disrupted Hela cells, which each contain (10) copies of integrated HPV DNA per cell. This is the lower limit of detection representative of HPV infection as guaranteed by the kit manufacturers. Samples containing less HPV DNA than that in the low positive control may not be detected by the test. Third, the specimen obtained for analysis may not be representative of the actual cervical pathology. As with the Pap smear, these specimens are subject to a variety of "sampling errors". While using a probe for human DNA can assure us that a sample of appropriate quantity was obtained, we can make no assumptions about whether the transformation zone or any potential lesions on the cervix were sampled. Unlike the Pap smear, with which the presence or absence of endocervical cells can be used to assess for adequate sampling, with this assay we are limited to an assessment of specimen quantity.

Finally, according to the kit manufacturers, bloody specimens may yield false negatives. Six of the specimens in this study were considered "bloody" at the time of testing. Only one of these yielded a positive result by ViraPap, but four of the remaining five had abnormal cytology on the accompanying Pap smears. Had they been less bloody, HPV DNA may have been detected in these specimens; were these samples to be eliminated from the false negatives, the false negative rate would improve to 28.6%.

A molecular diagnostic test should not be allowed to substitute for clinical judgement. There were two instances in this study in which a vaginal biopsy was obtained. In both cases, the subject had a normal Pap smear and a cervical biopsy without evidence of disease on the day of study, but a vaginal biopsy which showed disease--koilocytic atypia in one case, and mild dysplasia in another. Only one of these subjects had HPV DNA detected by ViraPap. While these are only two examples, these disparate findings suggest that HPV DNA may move freely enough within the genital tract (in desquamated cells, etc.) to be picked up by a swab at a location other than the infected site. These findings should also serve to caution the clinician against a reliance on molecular diagnostic tests as a substitute for other means of evaluation.

The results of this study are similar to those found in other studies comparing cytologic evidence of disease with the detection of HPV DNA by hybridization methods. Morse et al. conducted a study using high stringency dot blot hybridization to detect HPV DNA in specimens obtained from 164 cervical scrapes (Ayre spatula) obtained from 143 women (27). The authors of this study classified smears showing CIN separately from those simply showing evidence of viral infection.

HPV DNA was detected in 64 (39%) of the specimens, while cytologic features suggestive of HPV infection were found in 67 (41%) of smears. As was the case in the present study, the same specimens did not necessarily contain both cytologic and hybridization-based evidence of disease. Sixteen (10%) of the samples which had normal cytology were found to contain HPV DNA. Our study showed a substantially higher percentage of such cases (25%). One explanation for this discrepancy is that specimens in our study were obtained from a "high risk" population, with a higher expected disease prevalence. The subjects in Morse's study were not preselected to be "high risk", although the high rate of abnormal cytologic findings and HPV DNA detection may suggest otherwise. There were 19 cases in which there was cytologic evidence of HPV infection, but no HPV DNA detected by hybridization. This yields a false negative rate of 28%, which may be explained in part by the

fact that the investigators used probes to only three different types of HPV; types 6,16, and 18.

When the specimens showing CIN were considered, 30/40 (75%) were found to harbor HPV DNA; 5 contained HPV type 6, and 21 contained HPV type 16. The hybridization test seemed to be a good indicator of infection in this group, as 27/30 (90%) of the HPV DNA-positive specimens showed evidence of HPV infection.

Another study which compared findings on Pap smears to the results of an HPV DNA hybridization test used cervicovaginal lavage as means of collecting the specimen for hybridization studies (26). Burk et al. carried out a prospective study of 60 women referred to a colposcopy clinic because of a previously abnormal Pap smear. Following the collection of a Pap smear, the cervix was swabbed with a normal saline solution, followed by 5% acetic acid. The cervix was then lavaged with 7-8 ml. of saline solution, and the washings aspirated from the posterior vaginal fornix. The solution was centrifuged, and a DNA extraction was performed on the sedimented cells. Southern blot analysis was carried out, and the membranes were probed with HPV types 6, 11, 16, and 18.

On the day of study, 28/60 (47%) of the women had an

abnormal Pap smear, and 32/69 (53%) had normal cytology. 21/28 (75%) of patients with ClassII-IV smears had HPV DNA detected by Southern blot on cervicovaginal lavage specimens. Of the 32 women with negative Pap smears, 9 (28%) had HPV DNA detected. A retrospective review of the Pap smear results within a one year period for this group of patients revealed that 6/9 (67%) had had a Class III or Class IV smear in previous or subsequent specimens. Amongst the 30 patients who tested positive for the presence of HPV DNA, 21 (70%) were found to have cytologic abnormalities; HPV detection by Southern analysis on lavage specimens was predictive of a dysplastic cervical lesion in at least 95% of the patients who underwent colposcopically directed biopsy. Pap smears done on the day of study detected only 68% of these biopsy-proven dysplasias. There were 7 patients in whom HPV DNA was not detected, but whom were found to have ClassII-IV smears. A false negative rate of 25% can be calculated for the HPV DNA detection method using the Pap smear result as the indicator of disease. Among the Pap smears showing cytologic abnormalities,(ClassI-IV), typing revealed type 6 in 2 cases, type 11 in 2 cases, type 16 in 7 cases, type 18 in 5 cases, mixed infection with types 11 and 18 in 1 case, and 4 cases with an unidentified type.

McNichol et al. used the filter in situ hybridization method to detect the presence of HPV DNA in cellular specimens

obtained from the Ayre spatula and endocervical swab after preparation of the Pap smear (28). In a patient population of 98 women referred to colposcopy clinic for a previously abnormal Pap smear, the authors detected HPV DNA of types 6/11/16 or 18 in 79(81%) of the specimens. Only 30 (31%) of the women had HPV infection recognized by cytologic means. The authors conclude that filter in situ hybridization is far more sensitive than cytology as means of recognizing HPV infection, and recommend that such a method be used to augment present Pap smear screening.

Differences in study design make a direct comparison between the results from these studies and our own impossible. There were some similarities; Burk et al. and McNichol et al. selected a patient population very similar to that in our study. Like our study, all three of these studies were designed in a prospective fashion, and included some comparisons between cytology specimens and hybridization methods as a means of detecting HPV infection. However, in each study the methods of sample collection and hybridization were different, and this may have affected the results substantially. A comparison of the sensitivities of HPV DNA detection cannot be made directly between these investigations.

Despite the differences in methodology and patient

population, one finding has emerged consistently from these and other studies; the use of hybridization techniques has identified HPV DNA in a large proportion of specimens found to be abnormal by other means (cytology, histopathology, or colposcopy). In addition, HPV DNA has been found in a significant percentage of samples otherwise considered to be free of disease. The sensitivity of HPV detection is clearly not high enough to justify abandoning the Pap smear, but detection of HPV DNA may assist in the interpretation of an otherwise normal smear.

Typing

Determinations of the viral type found in different lesions has provided a great deal of information on the tissue tropism, prevalence, and apparent oncogenic potential of various forms of HPV. On the basis of such typing studies "high risk" and "lower risk" HPV types have been suggested. The clinical utility of knowing the viral type with which a patient is infected has not yet been established. The ultimate usefulness of this technique will be determined by whether or not a knowledge of viral type affects patient management. Conceivably, infection with HPV-16, the type most highly associated with cervical carcinoma, could lead clinicians to recommend that a patient pursue more frequent monitoring of cervical cytology, or further diagnostic measures to assure

the absence of cervical disease. On a research level, viral typing continues to be an important way to study the oncogenicity, and to follow trends in the epidemiology of this genus of viruses.

In this study typing was performed on the specimens which had produced a positive result by ViraPap. These 16 specimens were predominantly of types 16/18 (6), and types 31/33/35 (6). There was one specimen of type 6/11, two instances of coinfection with types from groups 16/18 and 31/33/35, and one specimen which was untypeable by the ViraType method.

There was some variation in the degree of cytologic abnormality associated with different viral groupings. The specimen typed as 6/11 had a corresponding Pap smear interpreted as koilocytic atypia. The specimens typed as 16/18 had corresponding smears read as negative (4), CIN2 (1), and CIN3 (1). Smears corresponding to the type 31/33/35 specimens were interpreted as negative (1), koilocytic atypia (2), ungraded CIN (1), CIN2 (1), and CIN3 (1). The coinfected specimens had corresponding Pap smears of CIN2 and CIN3.

The sample size in this study is too small to analyze for type associated trends. An investigation of viral types found in different grades and kinds of cervical malignancy would ideally select specimens in both a retrospective and

prospective fashion, have a large sample size, and use multiple probes with both high and low stringency conditions to allow for the identification of new viral types.

Polymerase Chain Reaction

All the specimens in this study were carried through the polymerase chain reaction using HPV type 16 specific primers. All the specimens which had been identified as type 16/18 by ViraType testing produced an E6 amplification product. This finding suggested that HPV type 16 was present in all six specimens, but provided no information concerning type 18. Restriction analysis with a type 16 specific enzyme demonstrated that the PCR products were in fact amplifications of the type 16 E6 ORF. While this confirms the specificity of the amplification primers used, no conclusions can be drawn regarding the additional presence or absence of type 18 in the specimens.

Four additional specimens which had not been previously identified as containing HPV DNA produced amplification products. Two of the samples produced strong signals equal in intensity to those of the known 16/18-positive specimens. One of these samples (#33) had tested negative with the ViraPap screen, subsequent typing was not done. This subject had a normal Pap smear on the day of study; a corresponding

cervical biopsy showed koilocytic atypia, and colposcopy had revealed a characteristic punctate lesion extending into the endocervical canal. Thus, this specimen had evidence of HPV-related disease by two other parameters, but had escaped detection by both cytologic methods and the ViraPap screening test.

Specimen #40 yielded a positive ViraPap result, but had not hybridized with any of the type specific probes in the ViraType kit, and was therefore classified as "untypeable". This subject had a nodular cervix on gross inspection, and histopathology described as poorly differentiated, invasive squamous cell carcinoma. Of note, the endocervical sample from this subject had been described as "bloody" at the time of collection.

Given the intensity of the signal produced by the amplification products from these two specimens, and the evidence of disease by other parameters, it is unlikely that contamination is responsible for these results. It is likely that the HPV DNA exists in episomal form in the first of these cases, and in an integrated form in the invasive carcinoma. The fact that amplification proceeded in both these cases suggests that the PCR is not limited in its ability to detect HPV DNA in its varied states.

Specimens #21, and #22 produced weakly positive signals after PCR. Neither of these specimens had tested positive by ViraPap, but both had evidence of disease by Pap smear, biopsy and colposcopy. It is possible that these are examples of contamination with PCR fragments. It is unlikely that contamination occurred by carry over of raw material from other specimens, as the specimens adjacent to these were negative by ViraPap testing and show no evidence of amplification by PCR.

One specimen typed as 31/33/35, and the kit control for type 6/11 both produced weakly positive amplification products. This is of considerable interest in light of the fact that comparable specimens (one positive for 6/11 and five others positive for 31/33/35) did not give positive results. While contamination must always be considered in explanation, it is also possible that small differences in the base pair sequences within the highly conserved E6 ORF are responsible for the failure or success in amplification amongst these samples. Such subtle variation might not be evident with hybridization techniques using essentially the entire genome, but may be asserted when specific oligonucleotide primers are used to recognize sequences.

As described earlier, investigators using the PCR have reported detecting HPV DNA in as many as 84% of cytologically

normal cervical smears, a majority of dysplastic smears, and 100% of cervical carcinomas (29). Our study is distinguished from this and other studies using PCR by the relatively few specimens found to be positive for HPV 16 DNA. Our results suggest that when careful technique is observed, and primers are designed with specificity to the type being sought, the polymerase chain reaction can be a sensitive confirmatory test following hybridization studies, and may detect additional cases missed by these methods. Whether this labor intensive test will become commonly utilized will depend in part on whether further studies establish that detection of HPV DNA by this means is clinically significant in the absence of positive hybridization studies. Subjecting specimens to the PCR with a panel of primers specific to the higher risk types (16, 18, 31, 33, and 35) may prove useful.

CONCLUSIONS

A number of investigators have described a history of previously normal Pap smears in patients presenting with invasive cervical carcinoma. Frequently, the patient had a normal Pap smear within a year of diagnosis. Although the decrease in mortality due to cervical cancer in the United States can be attributed in large part to this simple screening test, a proportion of women with cervical disease are clearly going undetected.

The association between infection with HPV and cervical cancer is very strong. Detailed investigation on the molecular level continues to add evidence for its role in oncogenesis, and as the likely etiologic agent in cervical cancer.

Hybridization methods and newer amplification techniques have demonstrated HPV DNA in cytology and histology specimens without any evidence of disease, as well as in all grades of CIN and invasive cancer. These detection methods provide means of investigating the epidemiology and pathogenesis of this virus. Most importantly, they allow for the identification of individuals with HPV infection, perhaps leading to closer or more frequent monitoring.

Until an etiologic role for HPV in cervical carcinogenesis is definitively established, the presence of the virus can at least be considered a marker for an increased risk of developing cervical disease. This study undertook to assess the usefulness of applying a hybridization test for detecting HPV in conjunction with the Pap smear, as compared to a reliance on the Pap smear alone.

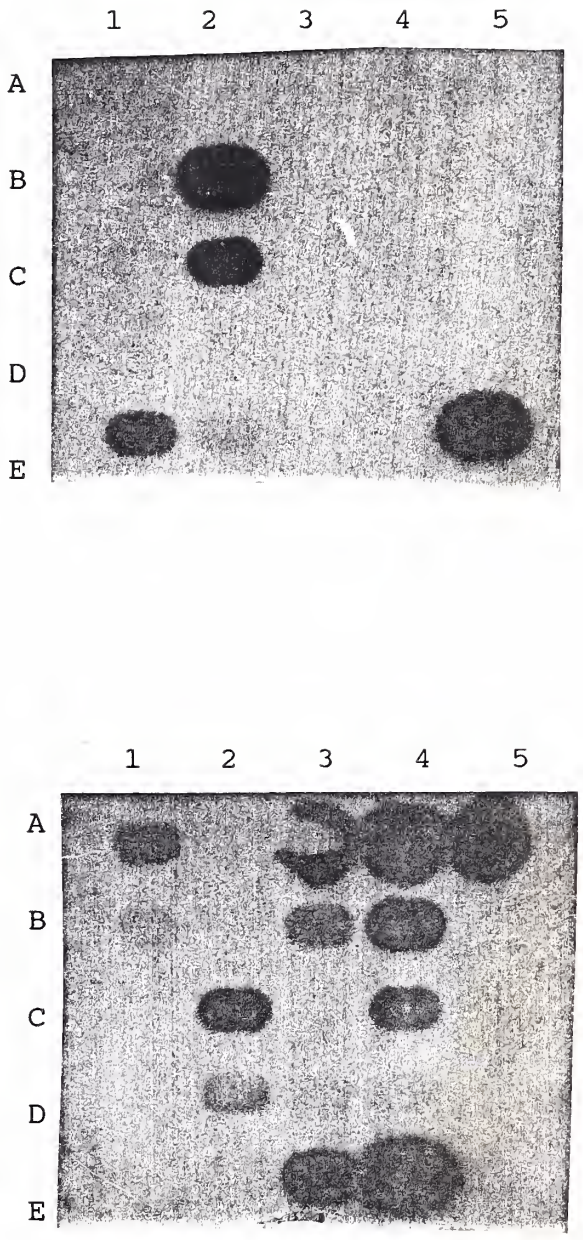
Our data showed that while cytologic examination revealed HPV-associated changes and dysplasias most frequently, the ViraPap detected HPV DNA in a significant proportion of the Pap smears not otherwise identified as abnormal. When the viral DNA present in these cases was typed, 80% of the cases were found to contain "high-risk" HPV types; the remaining case contained an HPV type associated with an "intermediate" risk of developing cervical disease. Given the fallibility of cytologic screening programs in the recognition of cervical malignancies and precursor lesions, we conclude that in a high risk population the use of an HPV detection test such as ViraPap serves as a useful adjunct to the Pap smear in identifying women at risk for developing cervical cancer.

APPENDIX

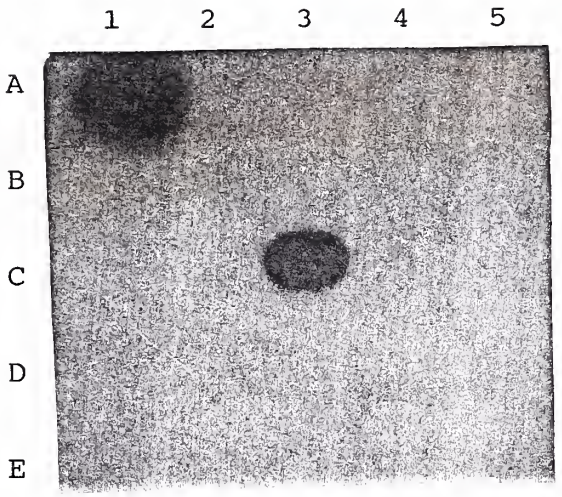
S#	Coord	VPap	Vtype	PCR	Pap	CBx	ECC	Colp	Preg
1	2(D1)	-	ND	-	-	ND	ND	-	-
2	2(E1)	-	ND	-	CIN3	ND	ND	+	-
3	2(A2)	-	ND	-	+	CIN2	ND	+	+
5	2(C2)/A2	+	31/33/35	-	CIN2	CIN3	CIN	+	-
6	2(D2)/A3	+	16/18	+	CIN2	CIN3	-	+	-
7	2(E2)/A4	+	31/33/35	-	+	CIN2	ND	+	+
8	2(A3)/A5	-	-	-	-	ND	ND	-	-
9	2(B3)/B2	+	31/33/35	-	+	+	ND	+	+
10	2(C3)	-	ND	-	+	+	+	+	-
11	2(D3)/B3	+	16/18	+	-	-(*)	-	+	-
12	2(E3)/B3	+	16/18	+	-	-	+	+	-
13	2(A4)/B5	+	16/18	+	-	ICC	ND	+	-
14	2(B4)/C2	+	31/33/35	-	+	ND	ND	+	+
15	2(C4)/C3	+	6/11	-	+	+	-	+	-
16	2(D4)/C4	+	16/18	+	-	ND	ND	+	+
17	2(E4)/C5	+	31/33/35 16/18	+	CIN2	CIN1	-	+	-
18	2(A5)/D2	+	16/18	+	CIN3	CIN3	ND	+	+
19	2(B5)	-	ND	-	-	ND	-	+	-
20	2(C5)	-	ND	-	-	-	-	+	-
21	2(D5)	-	ND	+	+	+	-	+	-
22	2(E5)/D3	-	-	+	+	+	-	+	-
23	1(A1)	-	ND	-	-	CIN1	-	+	-
25	1(C1)/D	-	-	-	+	CIN2	-	+	-
26	1(D1)	-	ND	-	-	-(*)	-	+	-
27	1(A2)	-	ND	-	-	CIN1	-	+	-
28	1(B2)/D5	+	16/18 31/33/35	+	CIN3	ND	ND	+	-
29	1(C2)/E1	+	31/33/35	-	CIN	ND	ND	+	+
30	1(D2)	-	ND	-	+	-	-	+	-
31	1(A3)	-	ND	-	-	ND	ND	+	-
32	1(B3)	-	ND	-	-	ND	-	+	-
33	1(C3)	-	ND	+	-	+	-	+	-
34	1(D3)	-	ND	-	-	ND	ND	-	-
35	1(A4)	-	ND	-	CIN1	ND	ND	ND	-
36	1(B4)	-	ND	-	-	ND	ND	ND	-
38	1(D4)	-	ND	-	+	+	+	-	-
39	1(E4)	-	ND	-	-	ND	ND	-	-
40	1(A5)/E2	+	-	+	+(@)	ND	ND	ND	-
41	1(B5)	-	ND	-	+	CIN1	ND	+	-
43	1(D5)	-	ND	-	-	ND	+	+	-
44	1(E5)/E3	+	31/33/35	+	-	+	-	+	-

Key: S# (Specimen number), Coord (Coordinates;refers to positions on ViraPap/ViraType filters), VPap (ViraPap), VType (ViraType), PCR

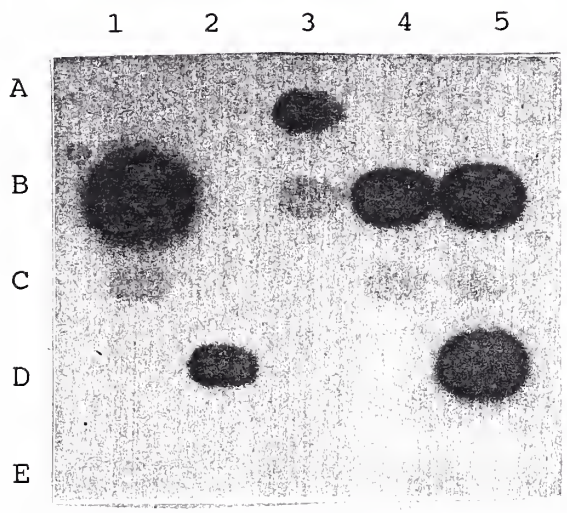
(Polymerase Chain Reaction), Pap (Papanicolaou Smear), CBx (Cervical Biopsy), ECC (Endocervical Curettage), Colp (Colposcopy), Preg (Gravity at time of specimen collection), ND (Not Done).
"+" under Pap, CBx, and ECC headings denotes koilocytosis or condylomatous changes.
(*) specimens #11 and #26 both had negative cervical biopsies but vaginal biopsies which showed CIN1 and koilocytosis respectively.
(@) specimen #40 did not have a Pap smear but
had a grossly nodular cervix, so was scored as positive.



(Figure 1) ViraPap Results. Filters #1 (top) and #2 (bottom). The high and low positive controls on filter #1 correspond to coordinates E1 and E2 respectively. On filter #2 high and low positive controls are at positions A1 and B1 respectively. For specific patient specimen information refer to the data listing in the appendix.

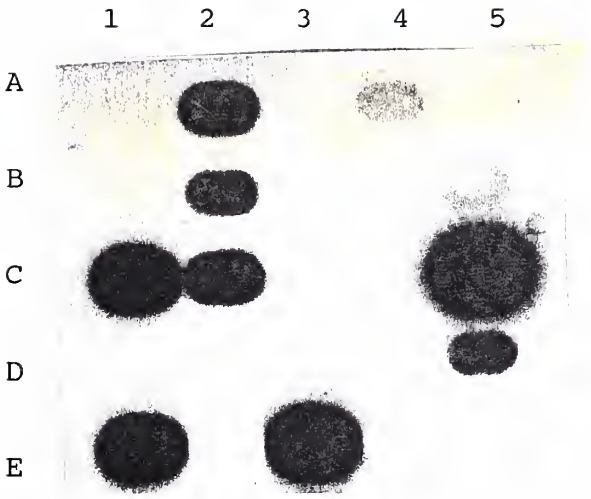


6/11



16/18

(Figure 2) See legend on following page.



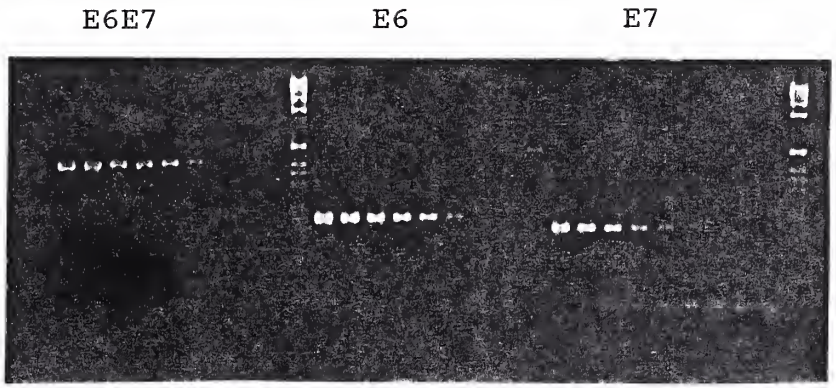
31/33/35

(Figure 2 continued) ViraType Results. Filters from ViraType procedure. Top filter probed with type 6/11 specific probes, middle filter with type 16/18 probes, and bottom filter with type 31/33/35 specific probes. Positive controls can be seen at positions A1, B1, and C1 on the 6/11, 16/18, and 31/33/35 respectively.

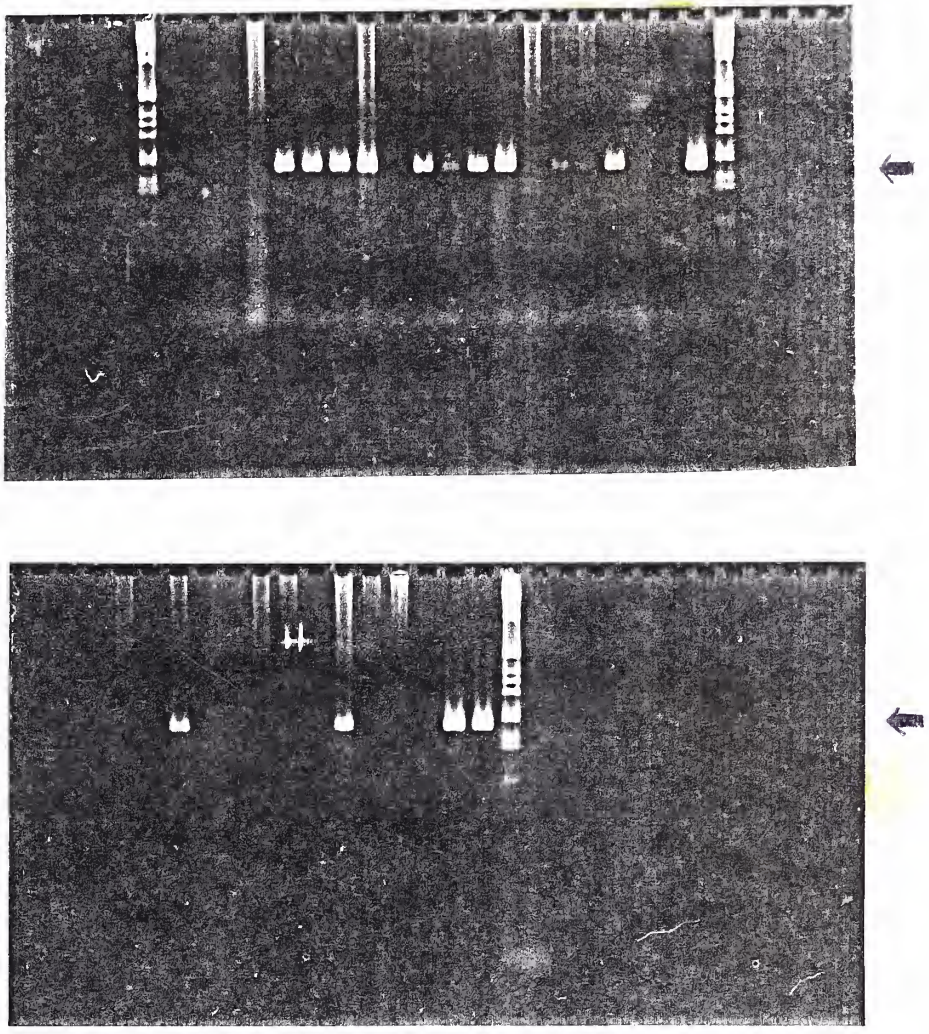
PCR PRIMER SEQUENCES

PRIMER		SEQUENCE		STARTING BASE PAIR
HPV16-1	5'	CAGCAATACAACAAACCGTTGTGTG	3'	371
HPV16-2	5'	GCTGGGTTTCTCTACGTGTTCTTG	3'	554
HPV16-3	5'	GCAACCAGAGACAACTGATCTCTAC	3'	606
HPV16-4	5'	GTACGCACAACCGAAGCGTAGAG	3'	769

(Figure 3a) Nucleotide sequences of the synthesized primers.

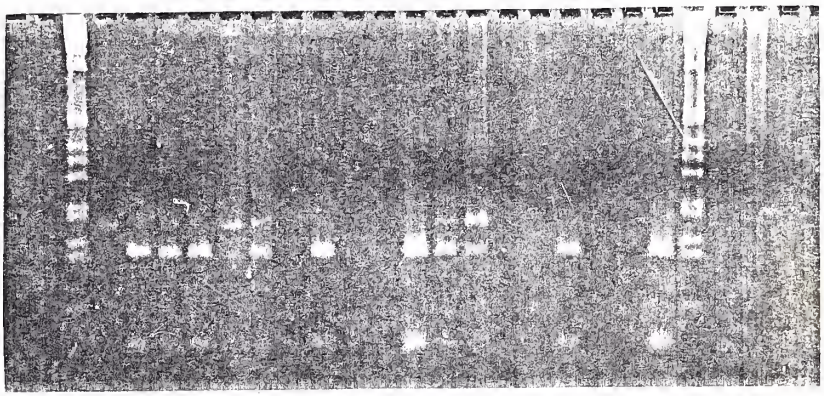


(Figure 3b) PCR products using serial dilutions of pBR322/HPV16 plasmid ranging from 1.0×10^{-2} to 1.0×10^{-8}. Primers HPV16-1 and 4, 1 and 2, and 3 and 4 were used to produce the E6E7, E6 and E7 products as shown. KB standard is at far right.

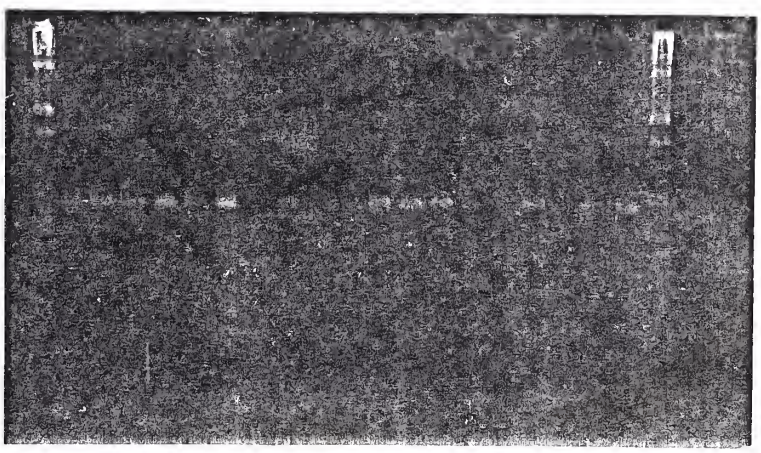


(Figure 4) PCR Results. HPV-16 E6 amplification of patient specimens using primers HPV16-1 and HPV16-2. Band corresponding to approximately 183 base pairs can be clearly seen at level of the arrow.

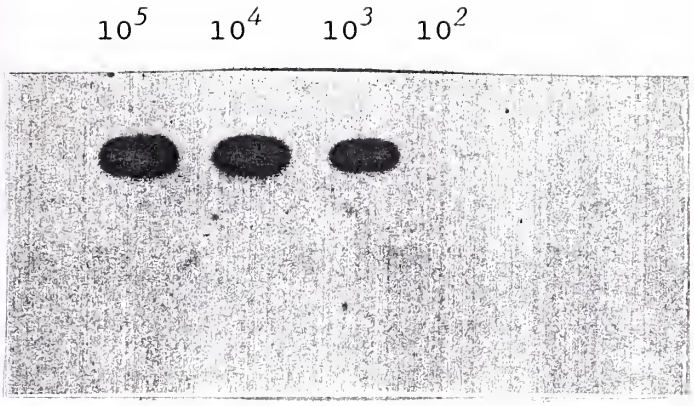
(a)



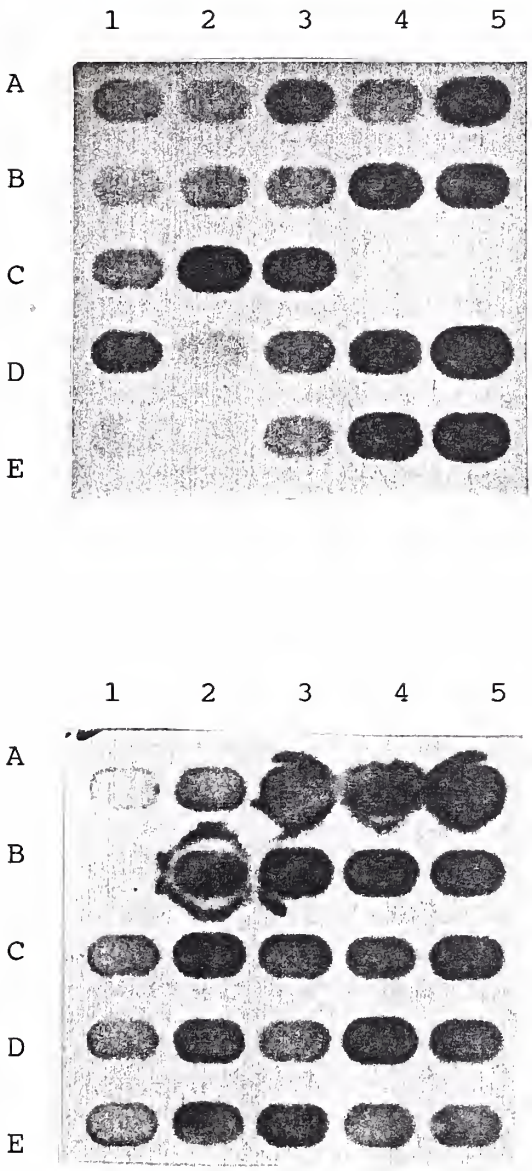
(b)



(Figure 5a and b) Restriction Digests. In (a) specimens cut with type 16 specific enzyme, TaqI. Remnants of uncut form are present at higher molecular weight. Cut form is visible as lower band. In (b), digested with a type 18 specific enzyme, Hae III, all the specimens remained in uncut form. There were no visible bands of lower molecular weight.



(Figure 6) Serial dilutions with labelled white blood cells.



(Figure 7) Probes for specimen adequacy. Original ViraPap filters were stripped and reprobed with random labelled human genomic probe. Filter #1 is at top, and filter #2 at the bottom.

REFERENCES

1. Davis GL, Hernandez E, Davis JL, Miyazawa K. Aytpical Squamous Cells in Papanicolaou Smears. Obstet Gynecol. 69(1):43-46, 1987.

2. Lunberg GD, ed. Toward Optimal Laboratory Use/Council Report: Quality Assurance in Cervical Cytology: The Papanicolaou Smear. JAMA. 262(12):1672-1679, 1989.

3. Bearman DM, MacMillan JP, Creasman WT. Papanicolaou Smear History of Patients Developing Cervical Cancer: An Assessment of Screening Protocols. Obstet Gynecol. 69(2):151-155, 1987.

4. Koss LG. State of the Art/Review: The Papanicolaou Test for Cervical Cancer Detection: A Triumph and a Tragedy. JAMA. 261(5):737-743, 1989.

5. Taylor PT, Andersen WA, Barber SR, Covell JL, et al. The Screening Papanicolaou Smear: Contribution of the Endocervical Brush. Obstet Gynecol. 70(5):734-737, 1987.

6. Lunberg GD, ed. Toward Optimal Laboratory Use: The 1988 Bethesda System for Reporting Cervical/Vaginal Cytological Diagnoses. JAMA. 262(7):931-934, 1989.

7. Robertson AJ, Anderson JM, Swanson Beck J, Burnett RA, et al. Observer Variability in Histopoathogical Reporting of Cervical Biopsy Specimens. J Clin Pathol. 42:231-238, 1989.

8. Salzman NP, Howley PM, eds. The Papovaviridae. Vol.2.: The Papillomaviruses. Plenum Press, New York, 1987.

9. de Villiers EM. Minireview: Heterogeneity of the Human Papillomavirus Group. J Virol. 63(11):4898-4903, 1989.

10. Koutsky LA, Galloway DA, Holmes KK. Epidemiology of Genital Human Papillomavirus Infection. Epidemiologic Reviews. 10:122-163, 1988.

11. Roman A, Fife KH. Human Papillomaviruses: Are We Ready to Type? Clin Micro Rev. 2(2):166-190, 1989.

12. Lowy DR, Schiller JT. Transforming Genes of Bovine and Human Papillomaviruses. In

13. Dyson N, Howley PM, Munger K, Harlow E. The Human Papilloma Virus--16 E7 Oncoprotein Is Able to Bind to the Retinoblastoma Gene Product. Science. 243:934-937, 1989.

14. Campion MJ, Singer A, Clarkson PK, McCance DJ. Increased Risk of Cervical Neoplasia in Consorts of Men with Penile Condylomata

Acuminata. Lancet. 943-946, April, 1985.

15. Tidy JA, Vousden KH, Farrell PJ. Relation Between Infection With A Subtype of HPV16 and Cervical Neoplasia. Lancet. 1225-1227, June, 1989.

16. Singer A, McCance DJ. The Wart Virus and Genital Neoplasia; A Casual or Causal Association. Commentary. Br J Obstet Gynecol. 92(11):1083-1085, 1985.

17. Venuti A, Marcante ML. Presence of Human Papillomavirus Type 18 DNA in Vulvar Carcinomas and Its Integration into the Cell Genome. J Gen Virol. 70:1587-1592, 1989.

18. de Villiers EM, Schneider A, Miklaw H, Papendick U, et al. Human Papillomavirus Infections in Women With and Without Abnormal Cervical Cytology. Lancet. 703-706, September, 1987.

19. Caussy D, Orr W, Daya AD, Roth P, et al. Evaluation of Mehods for Detecting Human Papillomavirus Deoxyribonucleotide Sequences in Clinical Specimens. J Clin Micro. 26(2):236-243, 1988.

20. Lorincz, AT. Human Papillomvirus Testing. Diag and Clin Testing. 27:28-37, 1989.

21. Melchers WJG, Schift R, Stolz E, Lindeman J, Quint WGV. Human Papillomavirus Detection in Urine Samples from Male Patients by the Polymerase Chain Reaction. J Clin Micro. 27(8):1711-1714, 1989.

22. Schneider A, Hotz M, Gissman L. Increased Prevalence of Human Papillomaviruses in the Lower Genital Tract of Pregnant Women. Int J Cancer. 40:198-201, 1987.

23. Horn PL, Lowell DM, LiVolsi VA, Boyle CA. Reproducibility of the Cytologic Diagnosis of Human Papillomavirus Infection. Acta Cytologica. 29(5):692-694, 1985.

24. Meisels A, Fortin R. Condylomatous Lesions of the Cervix and Vagina: Cytologic Patterns. Acta Cytologica. 20(6):505-509, 1976.

25. Day JD, Donaldson LD, Goellner JR. False-Negative Results in Cervical Cytologic Studies. Acta Cytologica. 29(6):1043-1046, 1985.

26. Burk RD, Kadish AS, Calderin S, Romney SL. Human Papillomavirus Infection of the Cervix Detected by Cervicovaginal Lavage and Molecular Hybridization: Correlation with Biopsy Results and Papanicolaou Smear. Am J Obstet Gynecol. 154(5):982-989, 1986.

27. Morse AR, Wickenden C, Byrne M, Taylor-Robinson D, et al. DNA

Hybridization of Cervical Scrapes: Comparison with Cytological Findings in Papanicolaou Smears. J Clin Path. 41:296-299, 1988.

28. McNichol PJ, Guijon FB, Paraskevas M, Brunham RC. Comparison of Filter In Situ Deoxyribonucleic Acid Hybridization with Cytologic, Colposcopic, and Histopathologic Examination for Detection of Human Papillomavirus Infection in Women with Cervical Intraepithelial Neoplasia. Am J Obstet Gynecol. 160(1):265-270, 1989.

29. Tidy JA, Parry GCN, Ward P Coleman DV, et al. High Rate of Human Papillomavirus Type 16 Infection in Cytologically Normal Cervices. Lancet. 434, February, 1989.

30. Barrett TJ, Silbar JD. Genital Warts--A Venereal Disease. JAMA.154(4):333-334, 1954.

31. White DO, Huebner RJ, Rowe WP, Traub R. Studies on the Virus of Rabbit Papilloma: Methods of Assay. Austral J Exp Biol. 41:41-50, 1963.

32. Beckmann AM, Myerson D, Daling JR, Kiviat NB, et al. Detection and Localization of Human Papillomavirus DNA in Human Genital Condylomas by In Situ Hybridization With Biotinylated Probes. J Med Virol. 16:265-273, 1985.

33. Stoler MH, Broker TR. In Situ Hybridization Detection of Human Papillomavirus DNAs and Messenger RNAs in Genital Condylomas and a Cervical Carcinoma. Human Pathol. 17(12):1250-1258, 1986.

34. Gage JR, Meyers C, Wettstein FO. The E7 Proteins of the Nononcogenic Human Papillomaviurs Type 6b (HPV-6b) and of the Oncogenic HPV-16 Differ in Retinoblastoma Protein Binding and Other Properties. J Virol. 64(2):723-730, 1990.

35. Bedell MA, Jones KH, Laimins LA. The E6-E7 Region of Human Papillomavirus Type 18 Is Sufficient for Transformation of NIH 3T3 and Rat-1 Cells. J Virol. 61(11):3635-3640, 1987.

36. Wilczynski SP, Walker J, Liao SY, Bergen S, et al. Adenocarcinoma of the Cervix Associated With Human Papillomavirus. Cancer. 62:1331-1336, 1988.

37. Skrabanek P. Cervical Cancer in Nuns and Prostitutes: A Plea for Scientific Continence. J Clin Epidemiol. 41(6):577-582, 1988.

38. McCance DJ, Clarkson PK, Chesters PM, Jenkins D, Singer A. Prevalence of Human Papillomavirus type 16 DNA sequences in cervical intraepithelial neoplasia and invasive carcinoma of the cervix. Br J Obstet Gynecol. 92:1101-1105, 1985.

39. McCance DJ, Kalache A, Ashdown K, Andrade L, et al. Human

Papillomavirus Types 16 and 18 In Carcinomas of the Penis From Brazil. Int J Cancer. 37:55-59, 1986.

40. Ostrow RS, Manias DA, Fong WJ, Zachow KR. A Survey of Human Cancers for Human Papillomavirus DNA by Filter Hybridization. Cancer 59:429-434, 1987.

41. Durst M, Kleinheinz A, Hotz M, Gissman L. The Physical State of Human Papillomavirus Type 16 DNA in Benign and Malignant Genital Tumors. J Gen Virol. 66:1515-1522, 1985.

42. Ismail, SM, Colclough AB, Dinnen JS, Eakins D, et al. Observer Variation in Histopathological Diagnosis and Grading of Cervical Intraepithelial Neoplasia. Br Med J. 298:707-710, March, 1989.

43. Cancer Facts and Figures-1989. American Cancer Society, New York, NY. 1989.

44. Shope RE. Infectious Papillomatosis of Rabbits. J Exp Med. 58:607-624, 1933.

45. Orth G, et al. Identification of Papillomaviruses in Butcher's Warts. J Invest Derm. 76:97-102, 1981.

46. Danos O, Giri I, Thierry F, Yaniv M. Papillomavirus Genome: Sequences and Consequences. J Invest Derm. 83(1):7s-11s, 198**.

47. Woodruff JD, Peterson WF. Condyloma Acuminatum of the Cervix. Am J Obstet Gynecol. 75:1354-1362, 1958.

48. Schneider V, Kay S, Lee HM. Immunosuppression as a High Risk Factor in the Development of Condyloma Acuminatum and Squamous Neoplasia of the Cervix. Acta Cytologica. 27(3):220-224, 1983.

49. Halpert R, Fruchter RG, Sedlis A, Butt K, et al. Human Papillomavirus and Lower Genital Neoplasms in Renal Transplant Patients. Obstet Gynecol. 68(2):251-258, 1986.

50. Sarver N, Rabson MS, Yang YC, Byrne JC, Howley PM. Localization and Analysis of Bovine Papillomavirus Type 1 Transforming Functions. J Virol. 52(2):377-388, 1984.

51. Phelps WC, Yee CL, Munger K, Howley PM. The Human Papillomavirus Type 16 E7 Gene Encodes Transactivation and Transformation Functions Similar To Those of Adenovirus E1A. Cell. 53:539-547, 1988.

52. Schneider-Maunoury S, Croissant O, Orth G. Integration of Human Papillomavirus Type 16 DNA Sequences: A Possible Early Event in the Progression of Genital Tumors. J Virol. 61(1): 3295-3298, 1987.

53. Smotkin D, Wettstein FO. Transcription of Human Papillomavirus Type 16 Early Genes in a Cervical Cancer and a Cancer-Derived Cell Line and Identification of the E7 Protein. Proc Nat Acad Sci. 83:4680-4684, 1986.

54. Fraumeni JF, Lloyd JW, Smith EM, Wagoner JK. Cancer Mortality Among Nuns: Role of Marital Status in Etiology of Neoplastic Disease in Women. J Nat Canc Insti. 42:455-468, 1969.

55. Towne JE. Carcinoma of the Cervix in Nulliparous and Celibate Women. Am J Obstet Gynecol. 69(3):606-613, 1955.

56. Harris RWC, Brinton LA, Cowdell RH, Skegg DCG et al. Characteristics of Women with Dysplasia or Carcinoma In Situ of the Cervix Uteri. Br J Cancer. 42:359-369, 1980.

57. Kessler II. Human Cervical Cancer as a Venereal Disease. Cancer Res. 36:783-791, 1976.

58. Martinez I. Relationship of Squamous Cell Carcinoma of the Cervix Uteri to Squamous Cell Carcinoma of the Penis Among Puerto Rican Women Married to Men with Penile Carcinoma. Cancer. 24:777-780, 1969.

59. Toon PG, Arrand JR, Wilson LP, Sharp DS. Human Papillomavirus Infection of the Uterine Cervix of Women Without Cytological Signs of Neoplasia. Br Med J. 293:1261-1254, 1986.

60. Daling JR, Weiss WS, Hislop TG, Maden C et al. Sexual Practices, Sexually Transmitted Diseases, and the Incidence of Anal Cancer. NEJM. 317(16)*;973-977, 1987.

61. Ferenczy A, Mitao M, Nagar N, Silverstein SJ, Crum CP. Latent Papillomavirus and Recurring Genital Warts. NEJM. 313(13):784-788, 1985.

62. Barnes W, Delgado G, Kurman RJ, Petrilli ES et al. Possible Prognostic Significance of Human Papillomavirus Type in Cervical Cancer. Gynecol Onc. 29:267-273, 1988.

63. Hadjimichael O, Janerich D, Lowell DM, Meigs JW, Merino MJ, Schwartz PE. Histologies and Clinical Characteristics Associated with Rapidly Progressive Invasive Cervical Cancer: A Preliminary Report From the Yale Cancer Control Research Unit. Yale J Bio Med. 62:345-350, 1989.

64. Lorincz AT, Temple GF, Kurman RJ, Jenson AB et al. Oncogenic Association of Specific Human Papillomavirus Types with Cervical Neoplasia. J Nat Canc Inst. 79(4):671-678, 1987.

65. Zachow KR, Ostrow RS, Bender M et al. Detection of Human Papillomavirus DNA in Anogenital Neoplasias. Nature. 300:771-773, 1982.

66. Campion MJ, McCance DJ, Cuzick J, Singer A. Progressive Potential of Mild Cervical Atypia: Prospective Cytological, Colposcopic, and Virological Study. Lancet. ii:237-240, 1986.

67. Ayre JE. Selective Cytology Smears for Diagnosis of Cancer. Am J Obstet Gynecol. 53:609-617, 1947.

68. Elias A. Linthorst G, Bekker B, Vooijs PG. The Significance of Endocervical Cells in the Diagnosis of Cervical Epithelial Changes. Acta Cytologica. 27:225-229, 1983.

69. Boons ME, Alons-van Kordelaar JJM, Rietveld-Scheffers PEM. Consequences of the Introduction of Combined Spatula and Cytobrush Sampling for Cervical Cytology. Acta Cytologica. 30:264-270, 1986.

70. Collins DN, Patacsil DP. Proficiency Testing in Cytology in New York: Analysis of a 14-Year State Program. Acta Cytologica. 30:633-642, 1985.

71. Gay JD, Donaldson LD, Goellner JR. False-Negative Results in Cervical Cytologic Studies. Acta Cytologica. 29:1043-1046, 1985.

72. Husain OAN, Butler B, Evans DMD, McGregor JF et al. Quality Control in Cervical Cytology. J Clin Pathol. 27:935-946, 1976.

73. Reagen JW, Wcott RB. The Detection of Cancer of the Uterine Cervix by Cytologic Study. Am J Obstet Gynecol. 62:1347-1352, 1951.

74. Richart RM, Vaillant HM. Influence of Cell Collection Techniques upon Cytological Diagnosis. Cancer. 65:1474-1478, 1965.

75. van der Graaf Y, Vooijs GP, Gaillard HLJ, Daisy M. Screening Errors in Cervical Cytology. Acta Cytologica. 31:434-438, 1987.

76. Tawa K, Forsythe A, Cove JK, Saltz A et al. A Comparison of the Papanicolaou Smear and the Cervigram: Sensitivity, Specificity, and Cost Analysis. Obstet Gynecol. 71(2):229-235, 1988.

77. Nyirjesy I. Atypical or Suspicious Cervical Smears. An Aggressive Diagnostic Approach. JAMA. 222:691-693, 1972.

78. Sandmere HF, Austin SD, Bechtel RL. Experience with 40,000 Papanicolaou Smears. Obstet Gynecol. 48:56-60, 1976.

79. Stafl A, Mattingly RF. Colposcopic Diagnosis of Cervical Neoplasia. Obstet Gynecol. 41:168-176, 1973.

80. Reiter RC. Management of Initial Atypical Cytology: A

Randomized Prospective Study. Obstet Gynecol.68:237, 1986.

81. Pilloti S, Rilke F, Shah KV, DelleTorre G et al. Immunohistochemical and Ultrastructural Evidence of Papillomavirus Infection Associated with In Situ and Micro-Invasive Squamous Cell Carcinoma of the Vulva. Am J Surg Path. 8(10):661-670, 1981.

82. Southern EM. Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis. J Mol Bio. 98:503-517, 1975.

83. Wilbur DC, Bontiglio TA, Stoler MH. Continuity of Human Papillomavirus (HPV) Type Between Neoplastic Precursors and Invasive Cervical Cancer: An In Situ Study. Am J Surg Path. 12(3):182-186, 1988.

84. Warford AL, Levy RA. Use of Commercial DNA Probes. Clin Lab Sci. 2:105-108, 1989.

85. Fetherston WC. False-Negative Cytology in Invasive Cancer of the Cervix. Clin Obstet Gynecol. 26(4):929-937, 1983.

86. Frenkel N, Roizman B, Cassai JE, Nahmias A. A DNA Fragment of Herpes Simplex 2 and Its Transcription in Human Cervical Cancer Tissue. Proc Nat Acad Sci. 69:3734-3789, 1972.

87. Sexually Transmitted Disease Statistics. United States Department of Health and Human Services. Public Health Service. Centers for Disease Control, Atlanta, GA., 1987.

88. Reid R, Greenberg M, Jenson AB, Husain M, et al. Sexually Transmitted Papillomaviral Infections: The Anatomic Distribution and Pathologic Grade of Neoplastic Lesions Associated with Different Viral Types. Am J Obstet Gynecol. 156(1):212-222, 1987.

89. ViraPap Test Kit Instruction Booklet. Bethesda Research Laboratories: Molecular Diagnostics Division. 1989.

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE